A STUDY OF BIOCHEMICAL AND MORPHOLOGICAL ASPECTS OF MACROPHAGE FUNCTION IN EXPERIMENTAL MURINE *NOCARDIA ASTEROIDES* AND *NOCARDIA BRASILIENSIS* INFECTIONS

by

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DEDICATION

For Bruce

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SUMMARY

A STUDY OF BIOCHEMICAL AND MORPHOLOGICAL ASPECTS OF MACROPHAGE FUNCTION IN EXPERIMENTAL MURINE *NOCARDIA ASTEROIDES* AND *NOCARDIA BRASILIENSIS* INFECTIONS.

Pathogenic *Nocardia* are facultative intracellular pathogens which are capable of growing within macrophages. Host resistance is believed to depend on interaction between activated macrophages and specifically sensitized T-lymphocytes.

The macrophage occupies a central position in the control of intracellular parasites by cellular immunity, and it has been established that initial interactions between *Nocardia* and macrophages are important in limiting the extent of infection that might develop.

Both specifically and non-specifically activated macrophages are important in the normal host defence against *Nocardia*. Although most studies suggest that macrophages are activated in *N.asteroides* and *N.brasiliensis* infections, it may be that an alteration in macrophage function occurs in association with the disease or as a result of corticosteroid treatment of the host. With these considerations in mind this study of macrophage function in experimental *N.asteroides* and *N.brasiliensis* infections was undertaken.

It had previously been noted that macrophage function in

N.asteroides and N.brasiliensis infections may be different. It was found that N.brasiliensis infection causes a depression in cellular immunity that affects the local host response.This study examines the possibility that macrophage responses in N.asteroides and N.brasiliensis infections respectively may be different by comparing biochemical and morphological aspects of macrophage function.

Murine macrophage function was determined at 2, 7, 13 and 21 days post-inoculation with live organisms of N.asteroides or N.brasiliensis and with normal saline. The macrophage function was assessed according to the release of plasminogen activator and lysozyme; and by morphology and ultrastructural study. The responses of macrophages to in vitro exposure to homologous culture filtrate antigens of Nocardia were determined.

The work has shown that there is early biochemical, morphological and ultrastructural change of peritoneal macrophages to inoculation with both N.asteroides and N.brasiliensis and that these responses do not differ in any major way in the two types of infection, although minor differences in enzyme release were shown at certain times post-inoculation.

It was found that plasminogen activator release by macrophages from mice previously inoculated with N.asteroides or N.brasiliensis was significantly greater at each time interval that was studied post-inoculation. Exposure to antigen in vitro did not significantly modify release of the enzyme. Macrophage

responses in N.asteroides and N.brasiliensis infections appear to be comparable with regard to the release of the enzyme, which was increased in both infections, although the degree of response differed at the different time intervals post-inoculation.

Lysozyme release, another index of macrophage activation, was examined in N.asteroides and N.brasiliensis infections. This was found to be less in macrophages from both N.asteroides and N.brasiliensis-inoculated mice harvested 7 days post-inoculation, and in macrophages harvested 13 days post-inoculation with N.brasiliensis and macrophages harvested 21 days post-inoculation with N.asteroides. Diminished secretion of lysozyme was associated with reduced intracellular levels, suggesting that reduced de novo synthesis rather than inhibition of enzyme secretion cause the reduced lysozyme levels in the extracellular medium. This aspect of macrophage response to the two Nocardia infections was different at 13 and 21 days post-inoculation.

Macrophages from N.asteroides- and N.brasiliensis-inoculated mice, and from controls were examined by phase-contrast and scanning electron microscopy. Macrophages from Nocardia -inoculated mice exhibited greater spreading, ruffled membranes, numbers of lysozomes, and pinocytic activity, which is characteristic of activated macrophages.

Macrophage responses to N.asteroides and N.brasiliensis infections were found to be comparable in respect of the parameters studied with the exception of lysozyme release at 13

and 21 days post-inoculation. In both infections macrophages are activated at each time interval post-inoculation studied with regard to release of plasminogen activator and morphology but not with regard to lysozyme release. This finding reinforces the idea that different conclusions regarding occurrence of activation of macrophages may be drawn depending on the criteria selected to assess this phenomenon. The finding that macrophages are activated in Nocardia infections with respect to certain parameters of macrophage activation is in keeping with the findings of others.

It is submitted in this thesis that the degree of activation or inhibition of macrophage function may differ in N.asteroides and N.brasiliensis infections with respect to release of plasminogen activator and of lysozyme . The pattern of secretion of plasminogen activator and lysozyme in N.asteroides infections appears to differ in N.brasiliensis infection; and there is possibly a difference in the amount of lysozyme released by 2 day N.asteroides-activated macrophages and 2 day N.brasiliensis -activated macrophages. Strains of Nocardia organism did not influence macrophage morphology or ultrastructure.

The study also shows the biochemical characteristics of plasminogen activator and lysozyme release, but not macrophage morphology and ultrastructure, are modified in the first 21 days of experimental Nocardia infections.

There are three apparent mechanisms by which virulent strains of

N.asteroides manage to survive within macrophages: (i) an ability to inhibit phagosome-lysozome fusion; (ii) alteration in the intraphagosomal pH; and (iii) alteration in the activity of the lysozomal enzyme acid- phosphatase. This study attempted to elucidate further the mechanisms enabling Nocardia organisms to persist and grow within macrophages. Reduced lysozyme release reflects diminished functional status of the macrophages of mice inoculated with N.asteroides or N.brasiliensis at certain times during infection. Reduced intracellular lysozyme levels have been linked with defects in bactericidal function. Such a reduction in intracellular and consequently extracellular levels of lysozyme might explain the capacity of Nocardia to survive intracellularly and to proliferate in the macrophage host.

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GLOSSARY OF ABBREVIATIONS USED

DB	Dulbeccos Modified Eagle Medium
TD	Tris Dulbecco's saline Ø.14M NaCl; 5mM KCl; Ø.7 mM Na_2HPO_4; 24.8mM Tris HCl_3; PH 7.4
TRIS	Tris (hydroxymethyl)-aminomethane
UK	Urokinase
BSA	Bovine Serum Albumin
FCS	Fetal Calf Serum
HIFCS	Heat-inactivated Fetal Calf Serum
ATFCS	Acid-treated Fetal Calf Serum
ATDS	Acid-treated Dog Serum
P+S	Penicillin and Streptomycin
M	Mycostatin
PMN	Polymorphonuclear leukocyte
MØ	Macrophage
CM	Conditioned Medium
PBS	Phosphate Buffered Saline
SGOT	Serum glutamic oxaloacetic transaminase

FIGURE KEY

C	=	Control macrophages
NA	=	N.asteroides-activated macrophages
NB	=	N.brasiliensis-activated macrophages
NA + Ø.ØØ2% AG	=	N.asteroides-activated macrophages exposed in vitro to Ø.ØØ2% antigen
NB + Ø.ØØ2% AG	=	N.brasiliensis-activated macrophages exposed in vitro to Ø.ØØ2% antigen
C*	=	Control macrophages exposed in vitro to Ø.Ø2% antigen
NA*	=	N.asteroides-activated macrophages exposed in vitro to Ø.Ø2% antigen
NA**	=	N.asteroides-activated macrophages exposed in vitro to Ø.ØØ2% antigen
NB*	=	N.brasiliensis-activated macrophages exposed in vitro to Ø.Ø2% antigen
NB**	=	N.brasiliensis-activated macrophages exposed in vitro to Ø.ØØ2% antigen
p	=	Level of significance, Mann Whitney U Test
MØ	=	Macrophage
T	=	Timepoint (e.g. T1, T2, T3)
S	=	Sample (e.g. S1, S2, S3)
mu UK	=	Milliunits of Urokinase

TABLE KEY

Ag	=	Antigen
C	=	Control macrophages
N	=	Nocardia-activated macrophages
Na	=	N.asteroides-activated macrophages
Nb	=	N.brasiliensis-activated macrophages
n	=	No. of replicates
conc	=	Concentration
M	=	Median
MØ	=	Macrophages
LL	=	95% confidence limit : Lower limit
UL	=	95% confidence limit : Upper limit
T	=	Timepoint (eg T1, T2, T3)
S	=	Sample (eg S1, S2, S3)
d	=	Days
p	=	Significance level using Mann Whitney U Test
MWU	=	Mann-Whitney U-Test
Sal	=	Saline
PA	=	Plasminogen Activator
PLG	=	Purified plasminogen
Con A	=	Concanavalin A
Fib	=	Fibrinolysis
L	=	Lymphocytes (eg L+ = present, L- = absent)
Thio	=	Thioglycolate-elicited macrophages
Inoc	=	Inoculation
Incu	=	Incubation
DA	=	Direct Assay
IA	=	Indirect Assay
Spleno	=	Splenomegaly
Hepato	=	Hepatomegaly
ATDS	=	Acid-treated dog serum
FCS	=	Fetal calf serum
HIAT	=	Heat inactivated acid treated
E	=	Experiment number
%C	=	Median enzyme value expressed as percentage control value
N.ast infect	=	N.asteroides infection
N.bras infect	=	N.brasiliensis infection
CM	=	Conditioned medium
ATFCS-P	=	Plasminogen-depleted fetal calf serum
ATDS-P	=	Plasminogen-depleted dog serum
2d Na	=	Mice inoculated 2 days previously with N.asteroides
2d Nb	=	Mice inoculated 2 days previously with N.brasiliensis
7d Na	=	Mice inoculated 7 days previously with N.asteroides
7d Nb	=	Mice inoculated 7 days previously with N.brasiliensis
13d Na	=	Mice inoculated 13 days previously with N.asteroides
13d Nb	=	Mice inoculated 13 days previously with N.brasiliensis
21d Na	=	Mice inoculated 21 days previously with N.asteroides
21d Nb	=	Mice inoculated 21 days previously with N.brasiliensis

SECTION 1

INTRODUCTION : LITERATURE REVIEW, METHODS AND GENERAL PRINCIPLES

CHAPTER 1

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Chapter 1

1.1 INTRODUCTION

Almost 100 years ago, Nocard first described "bovine farcy" - a granulomatous disease of cattle characterized by abscesses, sinus tracts, pulmonary disease and eventual death - after isolating an agent from lesions in cattle in Guadeloupe (Nocard, 1888). Trevisan created the genus Nocardia in 1889 and Nocard's isolate became the type species, Nocardia farcinica (Stanford, 1983). Considerable taxonomic confusion over the identity of the original isolate has reigned since the 1970's and it has recently been accepted that N.farcinica be replaced by N.asteroides as the type species. It has also recently been requested that N.farcinica be rejected on the basis that the only existing sample of Nocard's original isolate is N.asteroides (Stanford, 1983).

Two years after Nocard first recognized N.asteroides as a pathogen, the first human disease due to this organism was reported by Eppinger (1890); and the first human infection caused by N.brasiliensis was reported by Lindenberg in 1909. Human disease ascribed to Nocardia was reported infrequently during the next 50 years (Sen and Louria, 1980:336) and was considered rare in comparison to other infectious diseases - an impression which was probably erroneous. Over the past 25 years there has been a striking increase in the frequency of reports of infections due to Nocardia species (Cox and Hughes, 1975; Krick, Stinson and Remington, 1975; Folb et al, 1976; Simpson et al, 1981; Law and

Marks, 1982). There is no readily available reason for the increase in the incidence of the disease but factors responsible include greater awareness, more aggressive diagnostic approaches and improved medical surveillance, better laboratory isolation techniques, increased survival of compromised individuals and the increasing use of agents that promote development of opportunistic infections (Sen and Louria, 1980 : 336; Beaman and Sugar, 1983; Smego et al, 1983).

1.2 MICROBIOLOGY

Nocardia organisms were formerly considered to be fungi but are currently classified as bacteria belonging to the order Actinomycetales within the family Nocardiaceae Castellani and Chalmers (Buchanan and Gibbons, 1974 : 726). Organisms belonging to the genus Nocardia are Gram positive and either partially or completely acid-fast (Ziehl-Neelsen stain), aerobic, non-spore forming and grow as a mycelium of branching filamentous rods, most nocardiae producing aerial hyphae which fragment into short rods (Curry 1980; De Villiers 1978; Sen and Louria 1980 : 336; Stanford, 1983). Other families of the Order Actinomycetales include Actinomycetaceae, Mycobacteriaceae and Streptomycetaceae (Buchanan and Gibbons, 1974 : 657).

Currently, there are about 20 species of nocardiae on the list of approved bacterial names (Skerman, McGowan and Sneath, 1980) and four species are generally recognised as potentially pathogenic to humans. N.asteroides, N.brasiliensis, N.caviae and N.madurae (Smego and Gallis, 1984). The major pathogenic species in man

are N.asteroides, N.brasiliensis and N.caviae, in that order (Smego, Moeller and Gallis, 1983).

1.3 EPIDEMIOLOGY

1.3.1 HABITAT AND DISTRIBUTION OF THE NOCARDIAE

The nocardiae seem to live as saprophytes on grains, grasses and in water. They are indigenous inhabitants of the soil, where they play an important part in the biological change taking place; and of decaying vegetable matter. The organisms are worldwide in distribution (Wilson and Miles, 1964 : 500; Sen and Louria, 1980 : 336; Beaman, 1976). They are not part of normal human flora (Stevens et al, 1981) but may rarely be a saprophyte in the upper respiratory tract or the skin (Stevens, 1983). N.asteroides, N.brasiliensis and N.caviae seem to live in the soils of most countries, however, N.asteroides occurs more often in temperate climates and N.brasiliensis in tropical and subtropical areas. N.caviae has not been shown to have a specific geographic distribution (Beaman and Sugar, 1983; Smego and Gallis, 1984).

1.3.2 FACTORS INFLUENCING DISEASE IN MAN

1.3.2.1 INCIDENCE OF DISEASE

An epidemiologic study between 1972 - 1974 in the USA revealed that 83.3% of Nocardia sp. infections were caused by N.asteroides, 6.9% by N.brasiliensis and 2.9% by N.caviae, the other 6.3% were indentified only as Nocardia species. In

contrast, N.brasiliensis is isolated from more than 90% of patients with mycetoma in Central and South America (Smego, 1984). The "Beaman" study indicated that about 500 - 1000 cases of nocardial infections may be seen each year (Beaman et al, 1976), although others suggest that the incidence of disease is higher (Curry, 1980), since Nocardia masquerades as other diseases such as tuberculosis, systemic mycosis, carcinoma and pyogenic infections and may therefore go unrecognized (Smego and Gallis, 1984). The study revealed no geographic predominance for nocardial infections in the USA.

The pathogenic nocardiae are capable of causing overwhelming disease with a high mortality rate - a survey of 147 case reports (Presant, Wiernik and Serpick, 1973) showed a 38% mortality in localized infections and 81% mortality in disseminated infections. 85% of the cases in Beamans epidemiological study were severe pulmonary-systemic or "primary" central nervous system infections (Beaman et al, 1976) - other sites besides the lung serve as primary infection sites, especially in the compromised host. The brain and central nervous system are the most frequent sites of disseminated nocardial infection (Beaman and Sugar, 1983). In accord with the figures of Beaman et al (1976), 85% of cases in Europe and North America are pulmonary and/or systemic, with estimates that 45% will have systemic dissemination and 75-79% pulmonary involvement. 81-91% of human infections are due to N.asteroides (Stevens, 1983). Although Nocardia infections can be serious and life-threatening cure is often achieved with early diagnosis and treatment (Curry, 1980).

The idea that Nocardia organisms are pathogenic only for immunologically incompetent individuals is incorrect (Curry, 1980). Nocardia infections are associated with immunocompromised hosts and an underlying defect is present in 50-80% of cases (Law and Marks, 1982; Stevens, 1983). Instances of nocardial infections have been reported in individuals in whom no underlying disease or immunosuppressive therapy can be identified. 15% of cases reported by Beaman et al (1976) occurred in apparently healthy people. In the same series, Nocardia was considered to be an opportunist in 53% of the cases. This figure is similar to that of Williams, Krick and Remington (1976). N.asteroides is recognized most commonly as an opportunistic pathogen whereas N.brasiliensis produces disease most frequently in healthy people (Smego and Gallis, 1984). In their study, 28% of N.brasiliensis- associated disease occurred in compromised hosts.

1.3.2.2 PREDISPOSING FACTORS

Numerous predisposing factors have been associated with N.asteroides and N.brasiliensis infections. In the report by Beaman et al (1976), immunosuppressants, neoplasia and organ transplants were the commonest underlying factors. Localised lung disease such as tuberculosis and pulmonary alveolar proteinosis contribute to opportunistic nocardial infection (Beaman et al, 1976). Other underlying diseases include diabetes mellitus, systemic lupus erythematosis (Law and Marks, 1982); chronic granulomatous disease of childhood (CGD) and pre-existing pulmonary disease such as obstructive lung disease

(Stevens, 1983); mycosis fungoides (Smego and Gallis, 1984); hypogammaglobulinemia (Dolan, McCullough and Gibson, 1960) and malnutrition (Ballenger and Goldring, 1957).

In various series, a large number of individuals infected with nocardiae have been associated with diseases characterized by defects in cell-mediated immunity, such as Hodgkins disease (Sen and Louria, 1980 : 337) and CGD. Immunosuppression with corticosteroids is a common predisposing factor (Rosett and Hodges, 1978) and about 20% of nocardial infections have occurred in these patients (Sen and Louria, 1980 : 337).

Simpson et al (1981) studied nocardial infections in the immunocompromised host and several factors predisposing the transplant patient to Nocardia infections were identified by reviewing the literature. These include the intensification of immunosuppressive therapy during rejection episodes, multiple rejection episodes, quality of donor organ histocompatibility, granulocytopenia, hyperglycaemia, renal failure, hypogammaglobulinemia and schedule for administration of maintenance corticosteroids.

Systemic nocardial infections are often associated with impaired immunity (Hay, 1983). Individuals infected with N.brasiliensis with apparently normal immune defences most frequently had infections confined to skin and soft tissues whereas those with impaired host defences were predisposed to developing systemic rather than cutaneous disease (Smego and Gallis, 1984). The same

appears to be true for N.asteroides. Most cases of nocardiosis due to N.asteroides in healthy individuals have presented as localized pulmonary infection (Freese et al, 1963).

1.3.2.3 SEX PREDILECTION

In all series reviewed for this dissertation there was a marked preponderance of males infected with nocardiae. In Beaman et al's series (1976) men outnumbered woman about 3 : 1; according to Senn and Louria (1980 : 337) the male : female ratio is 2 : 1; in the study by Presant, Wiernik and Serpick(1973) 69% of the patients were male. Other studies showed a male preponderance (Law and Marks, 1982; Smego and Gallis, 1984) and it seems that overall, infections in males predominates over females 2-3:1 (Stevens, 1983). Although the sex predilection remains unexplained (Sen and Louria, 1980 : 337) suggestions have been that rather than an increased susceptibility in males, differences in occupation or environment may lead to greater exposure to these soil-dwelling organisms (Smego and Gallis, 1984).

1.3.2.4 AGE PREDILECTION

Nocardia infections most frequently occur midlife (Sen and Louria, 1980 : 337). In the study by Beaman et al (1976), most patients were between 21 and 50 years old although ages ranged from 3 to 83 years. Other series have quoted mean patient ages as 42 years (Smego and Gallis 1984) and 41.7 years (Presant, Wiernik and Serpick, 1973). However, nocardial infections appear to occur at any age and the midlife predisposition may be related

to age when diseases treated by transplantation or characterized by defects in CMI such as Hodgkins disease are contracted (Sen and Louria, 1980 : 337).

1.3.2.5 ROUTE OF INFECTION

Nocardia organisms gain access to and cause disease in the human body by two main routes - the skin or lung (Law and Marks, 1982; Hay, 1983). It has been suggested that entry may be gained via the gastrointestinal tract eg via contaminated food, but evidence for this route is inconclusive (Hay, 1983). Organisms, probably originating in the soil, enter most commonly via an airborne route or by traumatic inoculation (Stevens, 1983; Sen and Louria, 1980 : 336), the lung being the commonest primary infection site and accounting for 60-80% of cases of systemic nocardiosis (Smego, Moeller and Gallis, 1983). In the series described by Presant, Wiernik and Serpick (1973), the port of entry was the lung in 65% of cases, the skin in 25% and the intestine in 4%. Although most nocardial infections are caused by inhalation of contaminated dust, some lung infections result from invasion of the lung by aspirated organisms or from bloodstream infections (Beaman, Goldstein et al, 1978). It is not certain whether there is widespread exposure to Nocardia, or whether sub-clinical sensitization occurs commonly as a consequence. If widespread exposure, eg via inhalation, does in fact occur, development of disease or clearance of the organisms may depend on the immunological competence of the host, as well as on other factors (Hay, 1983). There is a possibility of direct or indirect patient-to-patient or fomite transmission

occurring occasionally (Sen and Louria, 1980 : 336; Stevens, 1983). There have been reports of clustering in paediatric cases (Cox and Hughes, 1975), in groups of compromised hosts (Palmer, Harvey and Wheeler, 1974; Stevens et al, 1981) and of nosocomial cases (Rosett and Hodges, 1978).

1.4 CLINICAL MANIFESTATIONS

N.asteroides, N.brasiliensis and N.caviae, the three commonly recognized pathogenic nocardiae, are capable of producing three distinct types of infection : pulmonary or systemic nocardiosis, actinomycotic mycetoma, or localized extrapulmonary suppurative infections which are generally confined to the skin and adjacent tissues (Beaman et al, 1976). Originally it was believed that N.asteroides was the only species that produced pulmonary-systemic nocardiosis and N.brasiliensis and N.caviae the sole agents of actinomycetoma (Hay, 1983). However it is now clear that N.asteroides can produce actinomycetoma and N.brasiliensis and N.caviae pulmonary or systemic nocardiosis (Beaman et al, 1976; Hay, 1983).

1.4.1 NOCARDIOSIS

Nocardiosis is described as "usually a pulmonary, less often a cutaneous, illness that can become a multisystem illness" (Curry, 1980). Invasive pulmonary infection is the most frequently encountered form of systemic nocardiosis and the main route of infection is via the respiratory tract (Hay, 1983). Nocardiosis may be an acute, subacute or chronic suppurative infection (Smego and Gallis, 1984), - the spectrum of nocardiosis ranges

from mild localized bronchopulmonary disease to extremely aggressive primary lung infections that disseminate haematogenously to the central nervous system, skin and less frequently to practically any organ in the body (Beaman et al, 1976; Smego, Moeller and Gallis, 1983; Law and Marks, 1982). Therefore nocardiosis has a highly variable clinical presentation, in addition it mimics a variety of other pulmonary infections (Beaman and Sugar, 1983). The commonest aetiological agent of nocardiosis is N.asteroides (Hay, 1983). Symptoms of nocardiosis vary widely, from none at all to malaise, cough, fever and occasionally pleuritic pain (Williams, Krick and Remington, 1976). Abscess formation is typical of the disease and frequently cases present with chronic suppurative pneumonia and bronchitis (Stevens, 1983). The clinical manifestation is generally a suppurative lesion (usually non-caseating granuloma) (Stevens, 1983). It has also been described as a "focal pneumonitis with little inflammatory response, though frank abscess formation often occurs with progressive involvement " (Curry, 1980). Chest roentrographs are highly variable and non-specific - features include small and large cavities, small and large abscesses, single and multiple nodules, segmental infiltrates, lobar infiltrates, necrotizing bronchopneumonia or lobar pneumonia, lobar pneumonia with bulging fissures, masses with central cavitation, pleural effusions, empyema and occasionally miliary patterns have been observed. Haemoptysis may occur. Extension of the pulmonary infection to the chest wall may lead to formation of sinus tracts. Leukocytosis and mild anaemia have been associated with the disease (Curry, 1980;

Sen and Louria, 1980 : 338). After pulmonary infection, central nervous system infection, manifested by non-capsulated abscess, is the next most common presentation in Europe and North America (Stevens, 1983; Sen and Louria, 1980 : 338).

1.4.2 CHARACTERISTICS OF GROWTH OF NOCARDIA ORGANISMS IN TISSUES

Growth characteristics of Nocardia organisms in systemic nocardiosis differ from their growth characteristics in mycetoma - organisms grow as slender branching filaments in nocardiosis, whereas in actinomycetoma the organisms are grouped into tight aggregates of filaments called grains, although the tissue reaction is similar (Hay, 1983).

1.4.3 MYCETOMA

Mycetomas are chronic, indurated, granulomatous masses localized to skin and underlying bone, that steadily become more severe over several months or years. Early in infection, the main sign is the formation of a firm subcutaneous swelling but development is slow and insidious and later multiple draining nodules and sinus tracts develop which discharge sulphur granules and serosanguinous fluid onto the surface of the skin. These granules are characteristic of the causative agent of the mycetoma. The abscesses become indurated and are surrounded by granulomatous inflammation. Spread of infection is usually by direct extension through the tissues and invasion of underlying muscle, bone and connective tissue occurs. Mycetomas have been described as a "fistulous tumour" (Conde et al, 1982). Although

swelling, suppuration and healing occur in a cyclic pattern, progressive development of the disease process occurs (Conde et al, 1982).

Mycetomas are thought to occur after wound contamination by Nocardia-containing soil and most frequently develop on the lower extremeties, usually the foot or leg, however they may develop in any region of the body (Beaman and Scates, 1981). In Mexico, mycetomas are found on the backs of farm labourers, who tend to carry loads of soil on their shoulders. Secondary invasion of the lung may occur by direct extension from these mycetomas (Beaman, 1976 : 387; Law and Marks, 1982; Curry, 1980; Hay, 1983; Smego and Gallis, 1984; Zlotnick and Buckley, 1980; Beaman and Scates, 1981; Gonzalez-Ochoa, 1962). N.brasiliensis is the most important etiologic agent of mycetoma in the Americas and it is also a causative agent in Africa, India and other tropical and subtropical regions (Smego and Gallis, 1984).

1.4.4 PRIMARY CUTANEOUS NOCARDIOSIS

The third form of Nocardia infection, primary cutaneous nocardiosis, follows a more acute course. Route of entry is thought to be by traumatic inoculation and in this disease the organisms grow in the branching filamentous form. Presenting signs include cellulitis, pyoderma or subcutanous abscesses, it may also present as a lymphocutaneous or sporotrichoid syndrome in which the initial infection site is acutely inflamed with cutaneous nodules, and secondary lesions are produced along the

course of the lymphatics. It is not certain whether N.caviae can cause primary cutaneous nocardiosis by inoculation, however, this form of infection caused by N.asteroides and N.brasiliensis is seen more and more frequently in the United States (Law and Marks, 1982; Hay, 1983).

1.4.5 PROGNOSIS

Factors affecting prognosis of nocardial infections were identified by Presant, Wiernik and Serpick (1973). High mortality was associated with three patient groups. (1) Poorest prognosis was associated with disseminated disease (80% mortality). (2) Pulmonary nocardiosis with underlying disease and concommitant immunosuppresive therapy or Cushing's disease (80-100% mortality, whereas in patients with underlying disease not receiving corticosteroid or antineoplastic therapy, mortality was 20%). (3) Acute disease with symptoms of less than three weeks duration. These authors also found that primary central nervous system (CNS) disease which disseminated was associated with 90% mortality rate. Other factors associated with poor prognosis include prior use of steroids, CNS involvement and inappropriate antibiotic therapy (Rosett and Hodges, 1978).

1.5 **DIAGNOSIS**

In order to diagnose Nocardia sp. as the etiologic agent of disease, cultural confirmation is necessary since staining characteristics and morphology cannot be used to differentiate Nocardia sp. from Actinomyces (Sen and Louria, 1980 : 338). Serologic and skin tests are becoming useful diagnostic tests but

because of antigenic cross-reactivity with organisms such as Mycobacteria false positive results may occur. Recently a complement -fixation test has been developed (Stevens, 1983).

Blood and cerebrospinal fluid rarely yield a positive Nocardia culture, even when non-capsulated abscesses are present (Sen and Louria, 1980 : 338, 339; Smego and Gallis, 1984). Falsely negative results may be obtained from sputum specimens and aggressive diagnostic procedures are advocated, especially when pulmonary infection in the compromised host is being investigated. Invasive procedures such as aspiration of cutaneous lesions, thoracentesis, transtracheal aspiration, bronchial brushing and skin or lung biopsy may be required (Sen and Louria, 1980 : 338; Krick, Stinson and Remington, 1975; Smego and Gallis, 1984). Since Nocardia organisms can take up to four weeks to form colonies, organisms may not be isolated and identified in the bacteriology laboratory because of the common practice of discarding culture plates after 48 - 72 hours, consequently appropriate measures to retain the plates must be taken (Stevens, 1983).

Diagnosis is aided by a high index of suspicion, particularly in the immuncompromised patient who has acute respiratory tract infection.

Presence of cutaneous Nocardia infection indicates the need to investigate the patient for other infected foci, especially if the patient is immunocompromised (Krick, Stinson and Remington,

1975; Rosett and Hodges, 1978).

1.6 TREATMENT

The mainstay of treatment of nocardial infections for the last forty years has been the sulphonamides and they remain the drugs of choice today, according to many literature sources. The usual dose required is 10 grams per day in divided doses. Although nocardiosis may be treated effectively, mycetoma is frequently refractory to treatment, although dapsone and long-term sulphonamides may be effective (Sen and Louria, 1980 : 339); Curry, 1980; Law and Marks, 1982).

Because of the known efficacy of the sulphonamides in nocardial infections and the synergistic action of sulphonamide and trimethoprim against various organisms, the combination appeared to be a promising one in the treatment of nocardial infections (Curry, 1980). Smego, Moeller and Gallis, (1983) assessed the efficacy of this combination and from the results of their study concluded that this combination should be considered the drug of choice. In this study the commercial fixed dosage tablets (80/400 mg trimethoprim and sulphamethoxazole) were given, the mean daily dosage being 8.2 tablets per day. However, it seems that there is controversy over the optimal trimethoprim-sulphamethoxazole combination ratio (Smego and Gallis, 1984; Curry, 1980). Synergistic effect depends on the sulphmethoxazole-trimethoprim ratio, the specific strain, inoculum and length of incubation (Bennett and Jennings, 1978) and failure of therapy has also been reported (Geiseler et al,

1979). From retrospective case evaluations, it appears that the drug combination offers advantages over sulphonamides only, in patients with severe metastatic nocardial infections (Smego and Gallis, 1984).

Many other antimicrobial agents have been used either alone or in combination in the therapy of Nocardia infections. Dapsone is reported to be useful in the treatment of infections due to N.brasiliensis eg mycetomas (Stevens, 1983). Ampicillin, amikacin, clindamycin, cycloserine, minocycline and erythromycin have been used successfully, but marked strain-to-strain variation in sensitivity makes in vitro sensitivity testing imperative (Curry, 1980 ; Smego and Gallis, 1984). Beta-lactam agents, eg the cephalosporins, cefamandole, cefotaxime, cefuroxime (Stevens, 1983) have shown in vitro activity and cefotaxime has been shown to be highly effective in a murine model of acute pulmonary nocardiosis (Sugar, Chahal and Stevens, 1983).

Duration of treatment has not been clearly defined but relapse after inadequate time of therapy is common. Smego and Gallis (1984) advise treatment for 3-12 months depending on clinical severity of disease, immune status of the patient and duration of time when there is risk of infection. Immunosuppressed patients on maintenance corticosteroids may benefit from prophylactic low-dose therapy after resolution of the nocardial infection.

Helpful adjunct therapy includes surgical drainage of abscesses, excision of abscess cavities and necrotic tissue and amputation (Smego and Gallis, 1984; Sen and Louria, 1980 : 339).

1.7 ANIMAL MODELS OF NOCARDIA INFECTIONS : INVESTIGATION OF PATHOGENESIS AND HOST RESPONSE

1.7.1 INTRODUCTION

Although a large variety of animals are susceptible to naturally acquired Nocardia infections, difficulties were experienced with initial attempts to develop a suitable, representative animal model of the disease and contradictory or conflicting results were obtained (Beaman and Sugar, 1983). Early workers reported that nocardiae were not pathogenic for mice or guinea pigs, others concluded that adjuvants were required for reliable results and enhanced virulence of the organisms, and others reported that guinea pigs were susceptible and died following infection (Beaman and Sugar, 1983). Beaman and Sugar have published a comprehensive review of naturally acquired and experimental nocardial infections in animals which includes a discussion of early experimental infections, and consequently this will not be repeated here (Beaman and Sugar, 1983). A recently published review of the interactions of N.asteroides in BALB/c mice with special reference to modulation of macrophage function and induction of specific T-cells (Beaman and Black, 1985) is also recommended for the interested reader.

A variety of animals have been used as models in the investigation of mechanisms of nocardial pathogenesis. Recently, experimental infections have been produced in guinea pigs (Sundararaj and Agarwal, 1978; 1977a; 1977b) and rabbits have been used as a source of unelicited and specifically activated alveolar macrophages (Beaman, 1977; Beaman and Smathers, 1976; Beaman, 1979; Davis-Scibienski and Beaman, 198Ø) but the vast majority of work investigating host resistance and pathogenesis of nocardial infections has been done using a mouse model (Folb, Jaffe and Altmann, 1976; Beaman et al, 198Ø; Beaman, Gershwin and Maslan, 1978; Beaman and Maslan, 1977; Folb, Timme and Horowitz, 1977; Beaman and Maslan, 1978; Beaman et al, 198Ø; Beaman, 198Ø; Rico et al, 1981; Ximenez et al, 198Ø; Conde et al, 1982). The laboratory mouse is particularly suitable for use as a model because there are numerous varieties such as immunodeficient strains, which are immunologically well defined and facilitate investigation into immune aspects of host response to the nocardiae (Beaman and Sugar, 1983).

1.7.2 ANIMAL MODELS OF NOCARDIA INFECTIONS

1.7.2.1 NOCARDIOSIS

A critical problem in establishing an animal model of N.asteroides infections was the requirement for addition of specific adjuvants to the inoculum. N.asteroides suspended in saline and introduced intraperitoneally frequently induced a benign infection, whereas addition of adjuvant increased the virulence and produced malignant infection (Uesaka et al, 1971;

Krick and Remington, 1975).

Acute and chronic progressive Nocardia infections were established in mice without the use of adjuvant after intraperitoneal inoculation by Beaman (1973) and Folb, Jaffe and Altmann (1976), and this permitted detailed study of the natural history and pathology of the infection.

Host response to and pathogenesis of pulmonary and systemic nocardiosis has been investigated using a murine model by several workers. Krick and Remington (1975) developed a mouse model using N.asteroides suspended in saline or mucin adjuvant and although the resultant intraperitoneal infection did not appear to resemble human nocardiosis they came to the very important conclusion that N.asteroides infection produces a population of activated macrophages and that cell-mediated immunity, functioning through these macrophages, plays a significant role in host defence against N.asteroides. This paper paved the way for numerous studies attempting to define the mechanisms of host response to invasion by N.asteroides. Recently, murine models of nocardiosis that have the essential clinical characteristics of the disease in humans have been developed using particular strains of organisms (Beaman and Sugar, 1983).

1.7.2.2 MYCETOMA

Gonzalez-Ochoa (1973), Gonzales-Ochoa and Kumiko-Hojyo (1967) developed a murine model of mycetoma with most of the clinical features of human disease associated with Nocardia. He produced

a typical mycetoma in the foot pad of the mouse after a single inoculation of N.brasiliensis without adjuvant. Folb, Jaffe and Altmann (1976) also produced a granulomatous lesion with a central mass of organisms forming a granule after single foot pad inoculation with N.brasiliensis without adjuvant. Beaman and Scates (1981) inoculated mice intravenously with N.caviae (no adjuvant) and produced a chronic mycetoma model, although the route of inoculation in this model has been criticized since the commonest site of mycetoma development in humans is in the feet (Conde et al, 1982). Conde et al (1982) induced characteristic mycetoma in an inbred mouse model without use of adjuvant. Histological features of the lesions were similar to those described by Folb, Jaffe and Altmann (1976); Melendro et al (1978) and Ximenez et al (1980). However, approximately 100-fold fewer Nocardia organisms were required to produce mycetoma in inbred mice in comparison to outbred mice (Folb, Jaffe and Altmann, 1976; Conde et al, 1982).

Several reports describe induction of mycetoma after foot pad inoculation with N.brasiliensis organisms in incomplete Freund's adjuvant (Melendro et al, 1978; Ximenez et al, 1980; Rico et al, 1981; 1982). Zlotnik and Buckley (1980) induced mycetoma in mice using N.brasiliensis in saline or in adjuvant and showed that development of mycetoma is accelerated by presence of adjuvant. Although the use of incomplete Freund's adjuvant increases the susceptibility of the host to infection in an undefined way, the histology of the lesions do not differ from that of naturally occurring lesions in humans (Rico et al, 1982).

1.7.2.3 CONTRADICTORY INTERPRETATIONS OF ANIMAL MODEL DATA

Animals models of Nocardia infections described in the literature differ and hence conflicting reports have been published, eg there has been considerable controversy regarding the relative pathogenicity of Nocardia organisms for mice (Uesaka et al, 1971; Gonzalez-Ochoa, 1973, Folb, Jaffe and Altmann, 1976; Beaman, 1973; Beaman, 1976 : 387). Factors contributing to these contradictory reports include difficulties in working with pathogenic nocardiae; lack of standardization of culture conditions in inoculum preparation; use of adjuvant in the inoculum; use of animals of different genetic backgrounds and consequently different susceptibilities to the organism; lack of standardization of parameters of virulence, eg some workers have used lethality as a parameter (Krick and Remington, 1975; Folb, Jaffe and Altmann, 1976; Beaman and Maslan, 1978); organ tropism, and different routes of inoculation. It is important, therefore, to be careful when comparing results from different animal models (Beaman and Maslan, 1977; Conde et al, 1982; Beaman and Sugar, 1983).

1.7.3 EXPERIMENTAL NOCARDIA INFECTIONS IN ANIMALS

1.7.3.1 IMMUNODEFICIENT MURINE MODELS

Several genetically immunodeficient strains of mice have been used to investigate in vivo mechanisms of host response to pathogenic nocardiae. Immunodeficient mice have also been produced by manipulation, such as thymectomy, lethal irradiation and immunosuppressive therapy.

1.7.3.1.1 Congenitally Athymic (nude mice)

In a histopathological study of lesions produced by N.asteroides and N.brasiliensis, Folb, Jaffe and Altmann (1976) noted microscopical features of special interest in the N.brasiliensis lesion : foamy macrophages and the presence of organisms within the cell cytoplasm. These features are suggestive both of the features noted in lepromatous leprosy in man and of systemic Mycobacterium bovis (BCG strain) infection in the immune-suppressed mouse. This finding lead to the suggestion that the pathological effects of N.brasiliensis could be related to inactivation of normal T-lymphocyte function by this organism (Folb, Jaffe and Altmann, 1976). The idea that disorders of cell-mediated immunity may be particularly important in the pathogenesis of Nocardia infections (Folb, Jaffe and Altmann, 1976) led to the initial attempt to define the role of the T-lymphocyte in normal immunity to nocardial infections and the results suggested that the T- lymphocyte is an essential component in normal host resistance to N.asteroides and N.brasiliensis infection (Folb, Timme and Horowitz, 1977).

The athymic nude (nu/nu) mouse functions as an excellent model for defining the roles of T-lymphocytes in host resistance to Nocardia since these mice are totally deficient in T-cell function (Beaman and Sugar, 1983). Several detailed studies investigating nude mouse responses to N.asteroides, N.brasiliensis and N.caviae infections (Folb, Timme and Horowitz, 1977; Beaman, Gershwin and Maslan, 1978; Beaman, Goldstein et al, 1978; Beaman and Scates, 1981; Deem, Beaman and Gershwin,

1982) have established that T-cells are critical in host resistance to systemic and chronic nocardial infections (Beaman and Sugar, 1983). Nude mice were initially more resistant to the acute phase of infection than their immunologically intact littermates, but after about 72 hours the nude mice showed increased susceptibility and at about 2 weeks post-infection were significantly more susceptible than their normal littermates (Beaman, Gershwin and Maslan, 1978; Beaman, Goldstein et al, 1978; Beaman and Scates, 1981; Beaman and Sugar, 1983). Nude mice were more susceptible to intravenous (Beaman, Gershwin, and Maslan, 1978) and intranasal challenge (Beaman, Goldstein et al, 1978) than their normal heterozygous littermates. Studies with a virulent strain of N.asteroides showed that T-cells were essential for effective host reponse against systemic infection (Beaman, Gershwin and Maslan, 1978) and that T-cells are important in pulmonary clearance and prevention of dissemination from the lung since nude mice cannot eliminate the intranasally administered bacteria (Beaman, Goldstein et al, 1978).

Nude mice did not generally develop the granulomatous lesion characteristic of murine mycetoma that is induced in immunologically intact mice by N.brasiliensis (Folb, Jaffe and Altmann, 1976; Folb, Timme and Horowitz, 1977; Ximenez et al, 1980) and it was suggested that T-lymphocytes have a central role in granuloma development (Folb, Timme and Horowitz, 1977). Others have shown that athymic mice were not able to develop typical mycetomatous lesions after infection with either N.brasiliensis or N.caviae. Rather, the nude mouse developed

purulent abscesses and aggressive systemic disease which resulted in mortality of the mice much earlier than the immunologically intact animals. After foot pad inoculation with N.asteroides, nude mice became systemically infected and died soon after infection, whereas littermate controls developed an acute footpad inflammatory response (Beaman, Gershwin and Maslan, 1978; Beaman and Scates, 1981; Beaman and Sugar, 1983).

In an attempt to further elucidate the inter-relationship between T-lymphocytes and the state of macrophage activation, athymic nude and control littermate BALB/c mice were pretreated with either Corynebacterium parvum (Propronibacterium acnes) to non-specifically activate macrophage function, or dextran sulphate 500 to suppress macrophage function (Beaman and Black, 1985). Sublethal doses of N.asteroides were administered intra-nasally and pulmonary clearance determined in pretreated and unmanipulated control mice. Dextran sulphate inhibited organism clearance from the lungs of heterozygous (nu/+) mice whereas pulmonary clearance of nude (nu/nu) mice did not appear to be affected. The authors suggested that alveolar macrophage activity in nude mice is either already maximally suppressed against Nocardia or that a functional T-cell population is required for the systemic effect of dextran sulphate. In contrast, non-specific macrophage activation by C.parvum markedly enhanced pulmonary clearance of Nocardia in both athymic and heterozygous littermate mice. These data suggest that T-cells are not required for the non-specific stimulation of anti-

nocardial lung clearance mechanisms; and that non-specific activation of macrophages may occur independent of T-cell function (Beaman and Black, 1985).

Conclusive evidence that T-cells are vital for adequate host-resistance to nocardial infection was supplied by Deem, Beaman and Gershwin (1982). These workers adoptively transferred specifically primed purified T-cells to nude mice and demonstrated increased resistance to a virulent strain of N.asteroides in these animals.

1.7.3.1.2 Hereditarily Asplenic (Dh/+) Mice

Hereditarily asplenic mice show a major age-dependent deficiency in T-cell function with the development of decreased immunoglobulin production (Beaman, Gershwin and Maslan, 1978; Beaman and Sugar, 1983). Young asplenic mice which have a decreased T-cell function are most susceptible to acute, systemic nocardial infections (Beaman, Gershwin and Maslan, 1978; Beaman and Scates, 1981). Further, those animals that survived the acute phase of infection showed a marked inability to clear Nocardia organisms from the body. Asplenic mice appeared to remain uninfected or always died before progressive mycetomas developed in response to N.brasiliensis and N.caviae (Beaman, Gershwin and Maslan, 1978; Beaman and Scates, 1981; Beaman and Sugar, 1983).

1.7.3.1.3 B-Lymphocyte Deficient Mice

CBA/N mice have an X-linked deficiency in B-lymphocyte function

and therefore have a poor antibody response to T-dependent and T-independent antigens, resulting in production of abnormally low levels of immunoglobulin M (Ig M) and IgG_3 antibody. They seem to have a functional T-cell population. The mating of CBA/N females with DBA/2 males results in CBD2/F1 males which are B-cell deficient, whereas the female littermates are immunologically intact (Beaman, Gershwin et al, 1982; Beaman and Sugar, 1983). Use of these genetically controlled B-cell deficient mice and their normal littermates as an animal model of Nocardia infections permits evaluation of the role of B-cells in the immune response. (Beaman, Gershwin et al, 1982). The B-cell deficient male mice were no more susceptible to N.asteroides infection than were the normal female control mice. Interestingly, the immunologically intact female mice were markedly more susceptible than their male littermates to intravenous challenge with a lethal dose of N.asteroides.

Preimmunized deficient and normal mice were similar in their ability to resist challenge with a lethal dose of N.asteroides; and both the groups of preimmunized mice seemed to have the same ability to develop a cell-mediated immune response to Nocardia. High antibody titres were measured in female mice but not in the B- cell deficient males. The increased susceptibility of normal female mice suggested that presence of antibodies against Nocardia are detrimental to the host and enhance the development of the disease (Beaman, Gershwin et al, 1982; Beaman and Sugar, 1983).

Rico et al (1982) investigated the effect of specific deletion of B-lymphocytes bearing receptors for Nocardia on the pathogenesis of nocardial infection. Lethally irradiated mice were reconstituted with spleen cells previously depleted of lymphocytes bearing specific receptors for a Nocardia extract (NE), ie these animals would not be able to produce antibody against NE but would produce all other immunoglobulins normally. Specific B-lymphocyte-depleted mice had less swelling of the inoculated foot and mycetomas healed whereas control mice reconstituted with whole spleen cells developed mycetomas and some spontaneously lost their limbs. The sera of specific B-lymphocyte depleted mice did not contain haemagglutinating antibodies to Nocardia whereas control mice sera contained these antibodies. Production of delayed-type hypersensitivity (DTH) reaction was positive in specific B-lymphocyte depleted mice indicating that induction of cell-mediated immunity to Nocardia was not affected.

1.7.3.1.4 Germfree Mice

These mice have been maintained in a germfree environment which isolates the host from the influences of exogenous microbial interactions and from interactions with its own normal microflora. Also, germfree mice are ideal for study of immune reactions against a single, specific external factor (Beaman, Gershwin et al, 1980).

The response of germfree mice to N.asteroides administered intranasally or intravenously was very different from that of

conventionally grown mice. The germfree mice were very much more susceptible to infection with N.asteroides than conventionally grown mice; germfree mice frequently died. The germfree mice did not appear able to inhibit rapid growing of the organism in brain, kidney, adrenals or lungs. Treatment of germfree animals with lipopolysaccharide (LPS) prior to infection abrogated this increased susceptibility and rendered these animals equally or more resistant than conventionally grown animals. Therefore resident microflora and exposure of the host to exogenous micro-organisms are important in increasing host resistance to Nocardia. The suggested mechanism of the enhanced host resistance is that resident microflora non-specifically and continuously activate the host defence system. The study further suggests that the state of macrophage activation and the development of cell mediated immunity are vital in host protection against systemic nocardial infections (Beaman, Gershwin et al, 1980; Beaman and Sugar, 1983).

1.7.3.1.5 T-cell depleted mice (B mice)

T-cell depletion of mice was accomplished by thymectomy at or before 4 weeks of age, followed by lethal irradiation (900R) and reconstitution with B cells by bone marrow transfer. B mice did not form antibodies after immunization with a T-dependent antigen, (Burro eythrocytes) and did not produce haemagglutinating antibodies to Nocardia. (Rico et al, 1981; 1982). These mice can be used to determine the role of B-cells and antibody in resistance to nocardial infections. Mycetomas were induced in B mice after footpad inoculation with

N.brasiliensis in adjuvant. T-cell depleted mice were far more susceptible to severe disease after nocardial infection than immunologically intact animals, and although the inflammatory response was less intense than in intact animals, the lesions were more destructive and spontaneous loss of infected legs occurred (Rico et al, 1981; 1982). These results confirmed the important role of T-cells in the immune response to Nocardia but did not elucidate whether the lack of T- cells or T-cell dependent antibody contributed to severe disease.

A previous study indicated that passive transfer of specific antibody, in the absence of cell-mediated immunity, did not modify the course of nocardial infection, in fact, the antibody seems to facilitate the growth of the micro-organisms and the progression of disease (Rico et al, 1981). These findings were confirmed in the 1982 study (Rico et al, 1982) in that specific antibody either passively administered to the host or the inoculation of antibody-coated Nocardia organisms favoured progression of the infection. Administration of antibody, whether by passive transfer or by coating of organisms, enhanced progression of disease in both normal and T-cell depleted mice. These experiments ruled out any role for antibody in protection against Nocardia. Direct T-cell participation in resistance was demonstrated in the experiments detailed under 1.7.3.1.3 (Rico et al, 1982). Elimination of the specific humoral response to Nocardia was accomplished by removal of specific B-cells. These animals mounted effective DTH reactions and completely controlled their nocardial infection, further supporting the

concept that antibody to Nocardia facilitate progression of disease and further establishing the critical role of cell-mediated immunity in protection against Nocardia (Rico et al, 1982).

1.7.3.1.6 Immunocompromised Host Models

Nocardiosis is a serious clinical problem in the immuno-suppressed patient (Section 1.3.2; Beaman and Sugar, 1983). Animal models of nocardiosis in the immunocompromised host have been developed. Murine susceptibility to infection with N.asteroides, N.brasiliensis and N.caviae is enhanced by administration of cortisone, which caused more extensive lesions and higher mortality (Mishra et al, 1973). Cyclophosphamide treatment of mice markedly increased their susceptibility to N.asteroides. The ability of the organism to grow in its target organ was enhanced and elimination of Nocardia from the organs was impaired. Cyclophosphamide modified host resistance to N.asteroides more effectively than cortisone, prednisolone or azothiaprine (Beaman and Maslan, 1977; Beaman and Sugar, 1983).

1.7.3.2 STUDIES IN GUINEA PIGS

Guinea pigs were inoculated with N.asteroides emulsified in incomplete Freund adjuvant (Sundararaj and Agarwal 1977a) or immunized with its ribonucleic acid protein (P-RNA) (Sundararaj and Agarwal, 1977b). Cell-mediated immunity (CMI) was shown to develop both during experimental infections and after immunization. Adoptive transfer of immune spleen cells from

infected or immunized donor guinea pigs improved guinea pig survival and decreased Nocardia organism counts in the tissues, indicating that CMI is important in protection against N.asteroides (Sundararaj and Agarwal, 1978). The use of outbred animals for adoptive transfer experiments has been criticized (Beaman, Gershwin and Maslan, 1978; Deem, Beaman and Gershwin, 1982).

A further study investigating mechanisms of protection of CMI showed that there was a marked decrease in the number of viable intracellular organisms in immune macrophages, indicating that CMI is mediated in part by activated macrophages. The protective role of macrophages in N.asteroides infection was further demonstrated by the adverse effects on survival and clearance of organisms from the tissues produced by administration of anti-macrophage sera (AMS) to guinea pigs. Adoptive transfer of immune spleen cells to normal guinea pigs increased the intracellular decrease in number of viable organisms, indicating that humoral immunity has little or no role in protection against N.asteroides (Sundararaj and Agarwal, 1978).

1.7.3.3 ANTIBIOTIC TESTING IN MURINE MODELS OF NOCARDIOSIS INDUCED BY N.ASTEROIDES

The efficacy of amikacin was evaluated in an acute lethality model and a chronic infection model and compared with the effect of sulphonamides. Amikacin significantly increased survival rate in the acute lethality model whereas sulphonamides did not; and amikacin and sulphonamide therapy significantly increased the

rate of resolution of abscesses in the chronic infection model. Overall, amikacin appeared to be more effective than sulphonamide under the experimental conditions employed (Wallace et al, 1979).

A study by Sugar, Chahal and Stevens (1983) demonstrated the efficacy of parenteral cefotaxime in the treatment of acute pulmonary nocardiosis in the mouse. The broad spectrum therapeutic activity of cefotaxime makes it an attractive agent as initial and long-term therapy in febrile immune-impaired hosts and it would provide coverage for possible nocardial infection in cases where the etiologic agent of disease may not initially be apparent (Sugar, Chahal and Stevens, 1983).

1.7.3.4 AMYLOIDOSIS IN EXPERIMENTAL NOCARDIA INFECTION

Repeated inoculation of mice with either N.asteroides or N.brasiliensis; or chronic progressive disease may lead to the development of secondary amyloidosis (Folb, Horowitz and Greenwald, 1977).

1.7.4. IN VITRO STUDIES

In vitro studies performed with the aim of elucidating the mechanisms of host resistance and pathogenesis of nocardial infections have concentrated on cellular aspects of the immune response, in particular Nocardia-macrophage interactions have been investigated (Bourgeois and Beaman, 1974; Beaman and Smathers, 1976; Beaman, 1977; Filice, Beaman and Remington, 1980; Davis-Scibienski and Beaman, 1980a, b, c; Beaman, 1979; Black et al, 1983; Black et al, 1985).

The association of defects in cell-mediated immunity and increased susceptibility to nocardiosis lead to studies on interactions of Nocardia with activated macrophages (Filice, Beaman and Remington, 1980).

1.7.4.1 INTERACTIONS OF N.ASTEROIDES WITH MACROPHAGES IN VITRO

The first reported studies of the interaction of N.asteroides with cultured macrophages established that the Nocardia are able to persist and grow intracellularly both in peritoneal macrophages (Bourgeois and Beaman, 1974) and in alveolar macrophages (Beaman and Smathers, 1976) and should therefore be considered facultative intracellular parasites (Beaman, 1976 : 389). The macrophage response towards Nocardia depended on the strain of Nocardia with which they came in contact (Beaman and Smathers, 1976; Beaman, 1977).

Normal rabbit alveolar and mouse peritoneal macrophages failed to kill virulent strains of N.asteroides over a three hour period of incubation. Upon prolonged incubation the organisms

grew within the macrophages and eventually destroyed them (Bourgeois and Beaman, 1974; Beaman and Smathers, 1976; Beaman, 1977; Beaman, 1979; Filice, Beaman and Remington, 1980). In contrast, specifically activated alveolar macrophages temporarily retarded growth of N.asteroides (Beaman, 1979). Non-specifically activated mouse peritoneal macrophages were able to kill quite substantial numbers of virulent N.asteroides organisms within 6 hours of incubation and to inhibit the intracellular growth of the remaining organisms (Filice, Beaman and Remington, 1980). Activated macrophages appear, therefore, to be important in host defence against Nocardia infection (Krick and Remington, 1975; Beaman, 1979; Filice, Beaman and Remington, 1980). From these results, peritoneal macrophages seem to be more proficient in killing Nocardia than alveolar macrophages, a finding confirmed by Black et al (1985). Killing of N.asteroides by alveolar macrophages was markedly enhanced by a combination of host factors from preimmunized animals. Maximal killing of organisms was achieved using specifically activated macrophages combined with lymphocytes from specifically primed lymph nodes, immune serum and alveolar lining material. Killing of Nocardia was negligible and similar to that of control macrophages when specifically primed lymphocytes were not included and decreased killing resulted when other components were omitted, indicating that activated macrophages alone are not adequate to kill phagocytosed N.asteroides and that specifically primed lymphocytes are important in that host defence (Davis-Scibienski and Beaman, 1980c).

Since the initial interaction of Nocardia with macrophages determines the extent of the resulting infection (Beaman and Smathers, 1976; Black et al, 1983) macrophage responses to Scibienski and Beaman (1980a), using rabbit alveolar macrophages, showed a correlation between relative virulence of strains of N.asteroides and their resistance to phagocytosis and intracellular killing. This correlation was also shown by Black et al (1983, 1985),in mouse peritoneal and alveolar macrophages. In the same study, there was a direct correlation between organism virulence, lack of macrophage-induced ultrastructural damage and inhibition of phagosome-lysozome fusion (Davis-Scibienski and Beaman, 1980a). A further study on the same strain of N.asteroides at different stages of growth established a correlation between the relative virulence of the phase of growth of the organism and the degree of inhibition of phagosome-lysozome fusion (Davis-Scibienski and Beaman, 1980b). Inhibition of phagosome-lysozome fusion was studied in alveolar, peritoneal, splenic and Kupffer cells from non-immune BALB/c mice. Effectiveness of inhibition of fusion was determined using virulent and less virulent strains of N.asteroides and the non-pathogenic yeast Candida krusei was used as a control. Both strains of N.asteroides differed significantly from Candida and inhibited phagosome-lysozome fusion to a greater extent than C.krusei. The virulent strain of N.asteroides was associated with the lowest degree of phagosome-lysozome fusion. In addition, the different strains of organisms induced different degrees of inhibition of fusion in the different macrophage populations (See Beaman and Black, 1985, for details). The

inhibition of phagosome-lysozome fusion may therefore be one of the mechanisms of nocardial pathogenesis (Davis-Scibienski and Beaman, 1980b).

Further studies attempting to elucidate the mechanism or intracellular events enabling virulent Nocardia organisms to persist and grow within macrophages showed that, in normal murine macrophages, relative virulence and resistance to killing by macrophages correlated with resistance to phagocytosis, in addition the intracellular level of activity of the lysozomal enzyme, acid-phosphatase was decreased by in vitro infection with live virulent Nocardia organisms and the greater the level of intracellular infection (in terms of number of organisms) the more the level of acid-phosphatase activity was reduced. Killed bacteria of the virulent strain or live organisms of a less virulent strain did not produce this effect. Live cells of the virulent strain reduced acid-phosphatase activity levels to a greater extent in alveolar macrophages than in peritoneal macrophages, which is consistent with evidence that peritoneal macrophages are more proficient killers of Nocardia than alveolar macrophages (Black et al, 1983). Subsequent work revealed that lysozomal acid-phosphatase activity correlated with normal and activated macrophage killing efficiency - increasing in macrophages that killed intracellular organisms and decreasing inversely with numbers of intracellular oganisms in macrophages unable to kill or inhibit Nocardia cells - and consequently acid phosphatase activity is an effective marker of macrophage ability to inhibit growth of or kill N.asteroides. It was also shown

that macrophage populations isolated from different anatomical sites, eg splenic, alveolar, peritoneal and Kupffer, differ in their nocardicidal activity and concommitant resistance to loss of acid-phosphatase activity. Also, the ability of different macrophage populations to kill or inhibit nocardial growth correlated with the organisms ability to inhibit phagosome-lysozome fusion (Black et al, 1985; Beaman and Black, 1985).

The possibility that Nocardia might be able to neutralize the acidification of the phagosome was investigated in BALB/c mouse peritoneal macrophages (Beaman and Black, 1985). Less virulent N.asteroides and the nonpathogenic yeast, Saccharomyces were unable to prevent acidification of the phagosome and did not effectively inhibit phagosome-lysozome fusion. However, the virulent strain of N.asteroides, which grew within macrophages, inhibited phagosome-lysozome fusion and effectively prevented acidification of the phagosome. Two important conclusions were drawn from these data : Virulent N.asteroides may survive and grow within macrophages by modifying phagosomal pH; and neutralization of acidification may be fundamental in altering the activities of some lysozomal enzymes, since in many cases enzyme activity, eg acid-phosphatase, is pH dependent (Beaman and Black, 1985).

These studies reveal that virulent Nocardia organisms are protected by a complicated series of evasive mechanisms - not only do they resist phagocytosis and inhibit phagosome-lysozome fusion, but they also reduce the efficacy of enzymatic attack

when fusion does occur (Black et al, 1983; Black et al, 1985; Davis-Scibienski and Beaman, 1980a,b).

1.7.4.2 IN VITRO STUDIES ON N.BRASILIENSIS AND MURINE MACROPHAGES

Peritoneal cells from normal or N.brasiliensis-infected mice were incubated in the presence or absence of N.brasiliensis antigen and then infected with Listeria monocytogenes to assess the listericidal ability of those macrophages. Exudate cells from Nocardia-infected mice have a significantly increased capacity to digest Listeria in the presence of specific antigen, indicating that macrophages are activated by N.brasiliensis. The authors suggested that macrophages are important in host defence to N.brasiliensis infection (Melendro et al, 1978).

1.7.4.3 INTERACTIONS OF N.ASTEROIDES WITH NEUTROPHILS AND MONOCYTES IN VITRO

Neutrophils (PMN) are prominent in sites of infection with Nocardia, which suggests that they may have a significant role in defence (Filice et al, 1980), however neutrophils are not sufficient to destroy the organisms since clinical infection progresses until effective antibiotic therapy is administered (Filice, 1985). Studies of the in vitro interactions of Nocardia with human peripheral blood monocytes (MN) showed that these cells were unable to kill significant numbers of N.asteroides despite phagocytosis of the organism, whereas S.aureus and L.monocytogenes were consistently killed by PMN and MN. These cells failed to kill N.asteroides despite the

occurrence of an oxidative metabolic burst (Filice et al, 1980). Investigation of the apparent resistance of N.asteroides to metabolites of the burst such as in vitro generated superoxide anion, H_2O_2, singlet oxygen, showed that the organism was indeed highly resistant and this resistance is thought to be due partially to its relatively high levels of intracytoplasmic catalase activity (Filice, 1983). Mechanisms of resistance to the oxidative metabolites of the metabolic burst were further elucidated by Beaman et al (1983; 1985). For a comprehensive review see Beaman and Beaman (1984). Beaman et al (1983) isolated and characterised a unique form of superoxide dismutase (SOD) produced by a virulent strain of N.asteroides, strain GUH-2. Non-pathogenic strains of Nocardia did not secrete this enzyme and the less virulent N.asteroides strain 10905 has less SOD and catalase activity than the virulent strain GUH-2 (Beaman et al, 1983; 1985). SOD catalyses the dismutation of superoxide anion to produce the more toxic hydrogen peroxide, H_2O_2. H_2O_2 is strongly microbicidal both alone or in combination, eg with myeloperoxidase . Catalase breaks down H_2O_2 (Beaman and Beaman, 1984). A combination of these enzymes could be expected to be highly protective against oxidative killing mechanisms. A previous study indicated that log phase cells of N.asteroides GUH-2 are about 1000 times more virulent than stationary phase cells of the same organism (Beaman and Maslan, 1978; Beaman et al, 1983). Also, resistance to phagocyte antimicrobial activities is altered at different phases of growth (Beaman, 1979; Beaman et al, 1985). The finding that rate of secretion of SOD per cell was much greater in the early phase of the growth

cycle than during the stationary phase could provide a partial explanation for the increased virulence during log phase. The SOD is selectively secreted into the medium and is associated with the outer cell wall of the organism and could be highly protective against superoxide radical toxicity associated with phagocytosing PMN (Beaman et al, 1983).

A further study investigated the role of this SOD and catalase in organism resistance to killing by PMN (Beaman et al, 1985). The amount of intracytoplasmic catalase activity present in the virulent strain N.asteroides GUH-2 was also found to be growth stage dependent, with maximal levels during the early stationary phase, ie when SOD levels are decreased. PMN were unable to kill N.asteroides GUH-2 at this stage of growth but were able to kill log phase organisms. Treatment of early stationary phase cells with purified antibody specific for surface-associated SOD enabled PMN to kill significant numbers of organisms. This killing was abrogated by chlorpromazine which inhibits the oxidative metabolic burst of PMN's, indicating that kill was achieved by oxidative metabolism.

Experiments were done in which exogenously added SOD or catalase partially protected the less virulent strain of N.asteroides 10905, which lacks surface-associated SOD and which is killed by PMN in vitro. Addition of SOD and catalase completely protected this less virulent strain from killing by PMN's. Further evidence for the protective nature of these enzymes was shown by the inability of PMN's to kill log phase cells of a highly

virulent mutant of GUH-2 which contains 7 times more catalase at this stage than the parent (PMN can kill log phase GUH-2). This study showed that there is a relationship between phase of growth, virulence, high levels of catalase, production of SOD and resistance of N.asteroides to PMN-mediated killings, and clearly demonstrates that SOD and catalase are important factors associated with virulence of Nocardia (Beaman et al, 1985).

In a further attempt to elucidate the role of PMN in host defence against Nocardia, Filice (1985) demonstrated that PMN inhibit the growth of N.asteroides in vitro. Most organisms were phagocytosed by PMN in vitro but most nocardiae did not appear to be damaged. Effects of PMN were inhibition of filament formation and amino acid uptake, also intracellular Nocardia did not become acid-fast. The oxidative metabolic burst was not needed for inhibition. Inhibition of filament formation continued for 7.5 hours and on further incubation the organisms elongated and escaped from inhibition. Addition of fresh neutrophils at 7.5 hours extended inhibition of filament formation and amino acid uptake. After about 20 hours of incubation nocardiae escaped from inhibition. Inhibition of growth may therefore occur in vivo mediated by attraction of fresh neutrophils into the infection site and consequently maintaining inhibition longer than the short-lived individual neutrophil is able. Consequently, neutrophils may be important early in infection before the development of activated macrophages (Filice, 1985).

A recent study investigated the hypothesis that synergy between

antimicrobials and phagocytes may occur during N.asteroides infection. Pre-incubation of nocardiae with various appropriate antimicrobials did not alter kill achieved by PMN and monocytes; similarly pre-exposure of organisms to PMN did not increase nocardial susceptibility to antimicrobials. Thus synergy between phagocytes and antimicrobials in host defence against N.asteroides was not shown (Filice and Fischer, 1986).

1.7.4.4 INTERACTION OF T-CELLS WITH N.ASTEROIDES IN VITRO

Deem, Doughty and Beaman (1983) demonstrated that macrophage-depleted populations of splenic lymphocytes from mice immunized with killed N.asteroides were able to kill N.asteroides organisms whereas lymphocytes from non-immunized mice did not. T-cells from spleens of immunized mice killed 80% of N.asteroides organisms within a 12 hour incubation period whereas macrophage-rich cell preparations from the same spleens killed only 40-50% of the organisms within the same time period. Preincubation of Nocardia-primed lymphocytes with nocardial antigen enhanced killing ability. Nocardicidal activity was enhanced in T-cell enriched populations and was eliminated by lysing lymphocytes with anti-Thy-1.2 plus complement, indicating that T-cells were responsible for the killing of N.asteroides. The microbicidal activity of N.asteroides-primed T-cells was immunologically specific and the mechanism of killing appeared to involve direct T-cell-Nocardia contact. This study underlines the importance of T-cells in the host-response to Nocardia and suggests that direct T-cell kill may be important in vivo since these organisms are frequently able to evade macrophage intracellular killing (Deem,

Doughty and Beaman, 1983).

1.7.5 OTHER FACTORS INFLUENCING HOST-PARASITE RELATIONSHIPS AND PATHOGENESIS

1.7.5.1 VIRULENCE

Several studies have established that the chemical and structural composition of the cell walls of Nocardia undergo significant changes during the growth cycle (Beaman, 1975; Beaman, Bourgeois and Moring, 1981; Beaman and Shankel, 1969; Beaman, 1973; Beaman and Sugar, 1983; Vistica and Beaman, 1983). Modification of the cell wall characteristics during the growth cycle has been correlated with dramatic changes in virulence of the organism and in host- parasite interactions (Beaman and Maslan, 1978; Vistica and Beaman, 1983; Beaman, 1979; Beaman, 1973; Beaman and Bourgeois, 1981; Beaman, 1976 : 388, 405; Davis-Scibienski and Beaman, 1980b; Beaman et al, 1985; Beaman, Bourgeois and Moring, 1981). An accurate and reproducible method for determining relative virulence of different strains of Nocardia was developed (Beaman, 1975; Beaman and Maslan, 1977; 1978). It was shown that a virulent strain of N.asteroides, GUH-2, was about 1400 times more virulent when injected intravenously into mice than the same organism injected i.v. in the stationary phase (Beaman and Maslan, 1978). All strains of N.asteroides, N.brasiliensis and N.caviae studied by these workers were more virulent in the log phase than the stationary phase of the same culture when inoculated intravenously into mice, however, degree of difference in virulence varied according to the strain being studied (Beaman and Sugar, 1983).

In contrast, Conde et al (1982) found that the virulence of N.brasiliensis did not vary during the different phases of growth. Beaman (Beaman and Sugar, 1983) used a different strain of N.brasiliensis and found that its virulence was related to phase of growth. These conflicting results were explained in terms of rate of growth of the strains of N.brasiliensis; and Beaman and Sugar (1983) concluded that growth rate but not relative size of individual cells, phase of growth and route of inoculation have a significant effect on nocardial virulence. In addition, it was shown that the type of media used to grow the organisms, as well as method of preparation of inoculum, had a profound effect on the degree of difference in virulence between stationary and log phase cells (Beaman and Maslan, 1978; Beaman and Sugar, 1983).

Extensive studies have been conducted in order to determine mechanisms of nocardial virulence and the effects of phase of growth on host-parasite interactions (Beaman, 1979; Davis-Scibienski and Beaman, 1980a, 1980b; Black et al, 1983; Beaman et al, 1983; Beaman et al, 1985; Beaman, Bourgeois and Moring, 1981; Filice, 1983; Vistica and Beaman, 1983). Earlier studies showed that normal macrophages were unable to kill virulent strains of Nocardia in vitro (Beaman and Smathers, 1976; Beaman, 1977). Incubation of virulent N.asteroides GUH-2 at different phases of growth with alveolar macrophages from normal and specifically immunized rabbits (Beaman, 1979; Davis-Scibienski, 1980b) revealed that nocardial cells from all phases of growth were able

to grow in normal alveolar macrophages, whereas activated macrophages successfully inhibited growth of stationary phase cells for at least 12 hours, but only temporarily slowed down growth of log-phase cells, which were significantly more toxic and antiphagocytic than stationary phase organisms (Beaman, 1979).

Interestingly, activated macrophages killed more log-phase cells than stationary phase cells at 3 hours after infection (Beaman, 1979) although log phase cells are more virulent (Beaman and Maslan, 1978). It appears that the filamentous or log form are initially more fragile and more easily killed than the coccoid form of the same culture although they are more virulent and grow more rapidly within macrophages (Beaman and Sugar, 1983). This surprising finding has been explained by Beaman et al (1985) who suggest that enhanced initial killing of log-phase cells, despite increased inhibition of phagosome-lysozome fusion (Davis-Scibienski and Beaman, 1980b) and reduced levels of lysozomal acid-phosphatase (Black et al, 1983), is related to oxygen-dependent rather than oxygen-independent microbicidal mechanisms. Intracytoplasmic levels of catalase are low during log-phase whereas SOD levels are high (Beaman et al, 1985) therefore it is conceivable that effects of H_2O_2 may be more lethal to log-phase organisms .

Degree of virulence of strains of N.asteroides as well as cell viability correlated with the ability of phagocytosed Nocardia organisms to inhibit phagosome-lysozome fusion and to resist

intracellular killing (Davis-Scibienski and Beaman, 1980a). Investigation of effect of phase growth of the virulent strain of N.asteroides GUH-2 established a further correlation between relative virulence associated with phase of growth and the degree of inhibition of fusion and suggested that this inhibition of phagosome-lysozome fusion may be one of the mechanisms of nocardial pathogenesis (Davis-Scibienski and Beaman, 1980b).

It appears likely that many aspects of nocardial virulence may be attributed to components that are either in the cell wall or on the cell surface (Davis-Scibienski and Beaman, 1980a; Vistica and Beaman, 1983; Beaman, 1976 : 407). The composition of the nocardial cell wall is structurally and chemically complex, consisting of several classes of free and bound lipids, proteins or peptides and polysaccharides such as trehalose dimycolates, nocobactins, peptidoglycan, arabinogalactan mycolates, nocardomycolic acids etc (Beaman and Beaman, 1984; Beaman, 1975); Beaman, 1976 : 402, 403; Beaman, Bourgeois and Moring, 1981). Trehalose dimycolate and sulpholipids are present in the cell envelope in mycobacteria and has been linked with pathogenicity of these organisms, also trehalose dimycolate is cytotoxic (Beaman, 1976 : 403; Vistica and Beaman, 1983). Mycobactins, a chemically similar compound to nocobatin, are found in the cell wall of mycobacteria and are considered to be a factor of mycobacterial virulence (Beaman, 1976 : 406). Trehalose dimycolate, sulpholipids and nocobactin may therefore play a role in nocardial virulence and host parasite interaction (Vistica and

Beaman, 1983).

A strain of N.asteroides, 14759, had almost twice the amount of muramic acid than glucosamine in its cell walls during the log phase compared with the stationary phase of growth (Beaman, 1975), ie the degree of polyunsaturation of muramic acid in both the alpha and beta portions of the molecule correlates with virulence (Beaman and Beaman, 1984; Beaman, Bourgeois and Moring, 1981). In addition, major changes occur in the size and molecular structure of the individual mycolic acids dependent on culture age. Stationary phase cells have mycolic acids of shorter length than the more virulent log phase of the same organism (Beaman, Bourgeois and Moring, 1981).

Vistica and Beaman (1983) attempted to further define cell wall components of nocardial pathogenesis. Comparison of cells of N.asteroides GUH-2 at different phases of growth showed a correlation between fatty acid composition, comparitive degree of cellular fragmentation from filamentous into coccobacillary form, and perhaps intracellular complexity with virulence of the organism.

Relative virulence of Nocardia and resistance to killing has been correlated with the ability of the organism to alter the intracellular activity of the lysozomal enzyme, acid phosphatase. The study showed that acid phosphatase levels increased or remained unchanged in macrophages phagocytosing less virulent or killed virulent nocardiae, but decreased in cells phagocytosing

live virulent organisms. This decrease in lysozomal enzyme level has been related to ability of virulent organisms to survive the action of the hydrolytic environment within the lysozome and it has been suggested that this ability contributes to the pathogenesis of Nocardia infection (Black et al, 1983).

The association of virulence and phase of growth of Nocardia with a cell-associated and secreted form of superoxide dismutase (SOD) as well as high levels of intracytoplasmic catalase has been discussed in section 1.7.4.3. Synthesis of these specific enzymes capable of neutralizing the toxic products of oxygen metabolism is likely to play a significant role in nocardial pathogenesis (Beaman et al, 1983; Beaman and Beaman, 1984; Beaman et al, 1985; Beaman and Sugar, 1983). Ability of Nocardia to resist oxidative metabolites is probably due to a combined interaction between catalase, SOD and the highly unsaturated lipids in the cell wall (Beaman and Beaman, 1984).

Virulence of nocardiae may not be attributed to any single specific mechanism but is the result of a complex combination of several unique properties (Beaman and Beaman, 1984). Factors associated with virulent strains of nocardiae include structure and composition of cell wall components, eg degree of polyunsaturation of mycolic acids as well as length of the individual mycolic acid chains, which is associated with phase of growth of the organisms, presence of SOD and catalase, alteration in the activities of the lysozomal enzymes, alteration of intraphagosomal pH and ability to inhibit phagosome-lysozome

fusion (Beaman, Bourgeois and Moring 1981; Black et al, 1983; Beaman et al, 1985; Davis- Scibienski and Beaman, 1980a, 1980b; Beaman and Black, 1985).

1.7.5.2 ROUTE OF INFECTION

Route of inoculation has a significant effect on the susceptibility of the host to Nocardia (Beaman et al, 1980a; Beaman et al, 1980b; Beaman, Maslan et al, 1980a; Beaman, Goldstein et al, 1978; Beaman and Sugar 1983). Virulent strains of N.asteroides and N.caviae were grown under identical conditions to early stationary phase and then inoculated into mice by five different routes. N.caviae 112 was 30 times more virulent than N.asteroides GUH-2 when administered intranasally, whereas N.asteroides was 10 times more virulent than N.caviae when inoculated intravenously, and the organisms were of similar pathogenicity when injected intraperitoneally. Host response to N.asteroides and N.caviae differed according to route of infection, mice being more susceptible to N.asteroides administered intravenously and least susceptible to this organism inoculated into the footpad or tail. N.caviae was most pathogenic via the intranasal route and least pathogenic when injected intraperitoneally (Beaman, Maslan et al, 1980a; Beaman and Scates, 1981). This study indicated that there is a compartmentalization of the host response to Nocardia (Beaman, Maslan et al, 1980a). In another study it was shown that intranasal administration of N.asteroides induces a more severe pulmonary infection than does aerosol administration of similar numbers of organisms, indicating that pulmonary defences cope

less well with intranasally administered N.asteroides (Beaman, Goldstein et al, 1978; Beaman and Sugar, 1983).

1.7.5.3 L-FORMS AND NOCARDIAL PATHOGENESIS

L-forms or L-phase variants are wall defective microbial variants capable of growth as non-rigid cells due to the absence of a rigid cell wall. They are able to grow continuously in their altered form and since these forms are gram-negative and pleomorphic they are very difficult to distinguish within lesions using conventional light microscopy. There are two types of L-forms : type A have no apparent cell wall whereas type B have the outermost cell wall layers but lack rigidity. Reversion to the parental organism may occur in L-phase variants but is not usual in true L-forms. (Beaman, 1976 : 398).

Structural and chemical changes occur in the cell wall during growth within the host (Beaman and Sugar, 1983; Beaman 1976 : 388). Beaman (1973) found that changes induced in the cell envelope of N.asteroides within host tissue were most dramatic in the least virulent strains. These observations lead to the finding that the nocardiae can live as facultative intracellular parasites and that altered forms of the organism (eg L-forms) seem to play a part in nocardial pathogenesis (Beaman, 1976 : 407). Bourgeois and Beaman (1974) showed decisively that mouse peritoneal macrophages induced altered forms of a strain of N.asteroides intracellularly, and these were found to be L-forms and transitional phase variants (Beaman, 1976 : 399).

Nocardial L-forms have been induced *in vitro* by incubating a number of strains of *Nocardia* in medium containing glycine plus lysozyme to induce formation of spheroplasts in cultures from which L-forms could subsequently be isolated (Bourgeois and Beaman, 1976); and by incubating *N.asteroides* with cultured peritoneal macrophages (Bourgeois and Beaman, 1974) and alveolar macrophages (Beaman and Smathers, 1976). In both these latter studies, organisms disappeared within *in vitro* cultured macrophages, however some cells were able to avoid being killed - since the resulting protoplasts were protected from lysis by the macrophages (Beaman, Bourgeois and Moring, 1981) - and persisted as wall defective variants which ultimately replicated within the macrophages and killed them. During this time typical nocardial cells could not be recovered (Bourgeois and Beaman, 1974; Beaman and Smathers, 1976; Beaman, 1980).

There are a number of reports in the literature of initial failure to isolate *Nocardia* or microscopically demonstrate organisms in infected tissue, although clinical material obtained later or at autopsy was shown to contain organisms. In addition, although *Nocardia*-like organisms could be visualized in the tissue, standard culture conditions did not yield positive cultures. (Beaman, 1976 : 397; Krick, Stinson and Remington, 1975; Sen and Louria, 1980 : 338; De Villiers 1978; Smego and Gallis, 1984). There have been reports of slow progressive nocardial infection that developed after a long period of latency (Greer, 1974; Beaman, 1976 : 397), as well as reports of relapse of nocardial infection after clinical cure has apparently been

achieved (Stropes, Bartlett and White, 1980). An early study by Uesaka et al (1971) suggested the presence of an altered gram-negative form of Nocardia in organs of mice, since organisms could be isolated from tissues, whereas no organsism could be visualized in tissue sections. Intranasally administered N.caviae induced an acute pneumonic reaction and death in mice, and although macroscopic examiniation of these lungs revealed consolidation and abscess formation, few or no bacteria were isolated from lung homogenates (Beaman, Maslan et al, 1980a; Beaman, 1980). In these reports of human and animal infections it is possible that Nocardia organisms were present in the tissues in the form of altered variants which were induced in vivo (Beaman, 1976 : 397).

Studies investigating the induction of L-forms and L-phase variants in vivo have shown that L-phase variants are induced within the lungs of normal mice after intranasal administration of N.caviae 112 (Beaman, 1980), and that macrophages are capable of removing the cell wall of N.caviae 112 in vivo (Beaman, 1980; Beaman and Scates, 1981). It has been suggested that the mechanism of induction of L-phase variants and L-forms by macrophages in vivo may be similar to the i.v. in vitro induction of glycine and lysozyme (Bourgeois and Beaman, 1976; Beaman, 1980).

The induction of L-forms and L-form variants of N.caviae and probably other nocardiae appears to play an integral part in nocardial pathogenesis (Beaman and Sugar, 1983; Beaman, 1980;

Beaman and Scates, 1981). The presence of large numbers of L-phase variants in infected lung tissue at a time when pulmonary consolidation and death are occurring and normal bacteria cannot be isolated suggests that these L-phase variants play a major role in the infectious process and that these variants should be considered an alternate pathogenic form (Beaman, 1980). L-forms and L-phase variants appear to be involved in the latency of infection and in bacterial persistence (Beaman, 1980; Beaman and Scates, 1981; Beaman and Sugar, 1983). Studies with N.caviae showed that a cell wall-deficient form of N.caviae continued to exist in host tissues during the latent period between acute and chronic infection. The ability of these forms to persist for prolonged periods was demonstrated by the discovery that L-forms and L-form variants were present in mouse tissues (eg blood, liver, lungs and spleen) for more than a year, whereas no normal organisms were isolated from these organs (Beaman and Scates, 1981). Further, this study revealed that induction of L-forms occurs within immunologically competent mice but not in T-cell deficient animals, and suggested that L-forms of N.caviae are involved in the induction and formation of mycetomatous lesions. Mice injected intravenously with N.caviae that survived the acute phase of infection developed characteristic mycetomas almost a year later. Light microscopy of sections of mycetomatous lesions showed that granules contained L-forms of N.caviae. It was suggested that replication of these wall-deficient cells to form enlarging aggregates would lead to development of a host response, reversion of some L-forms to the walled state and growth of these revertants at the periphery of the aggregate and

development of the mycetomatous lesion (Beaman and Scates, 1981).

Removal of the nocardial cell wall either *in vitro* or *in vivo* can result in mutational alterations in the cell wall when it redevelops, which lead to altered cellular and colonial morphology (Beaman, Bourgeois and Moring, 1981; Beaman and Bourgeois, 1981; Beaman and Sugar, 1983). It was found that the longer the organisms persisted in a wall-less state, the greater the degree of cell wall modification during reversion (Beaman, Bourgeois and Moring, 1981). These observations suggest a mechanism for taxonomic heterogenecity among strains of *Nocardia* (Beaman and Bourgeois, 1981).

1.7.5.4 ROLE OF CELL-WALL ASSOCIATED LIPIDS IN THE PATHOGENESIS OF THE LESIONS

A recent study by Miller-Hardy and Reynolds (1984) investigated the role played by cell wall-associated lipids of *N.asteroides* and *M.fortuitum* in the pathogenesis of nocardiosis and mycobacteriosis produced by these pathogens respectively. Although these two organisms have similar cell wall-associated lipids they produce a distinctly different tissue response ranging from the acute suppurative lesion of nocardiosis to the granulomatous disease caused by *M.fortuitum*. Lipid fractions were separated, inoculated into mice and lesion development monitored. Wax A from *Nocardia* produced multiple abscesses whereas wax A from *Mycobacterium* caused a mild, transient reaction; Wax D from *Mycobacterium* caused a marked granulomatous

response, whereas Nocardia-derived Wax D produced a minimally acute inflammatory response. The authors suggested that the wax fractions A and D are responsible for the different tissue responses characteristic of these two infections (Miller-Hardy and Reynolds, 1984).

1.7.6 ROLE OF THE HUMORAL IMMUNE RESPONSE

Recent studies have revealed that the humoral immune response, and B-lymphocytes play little role in host defence against nocardial infections (Beaman et al, 1982; Ximenez et al, 1980: Rico et al, 1981; Rico et al, 1982; Conde et al, 1983; Beaman and Sugar, 1983). Further, it has been demonstrated conclusively that the presence of antinocardial antibody produced by B-cells can facilitate progression of disease and is deleterious to the host (Rico et al, 1981; Rico et al, 1982; Beaman et al, 1982; Conde et al, 1983; Beaman and Sugar, 1983). Rico et al (1981, 1982) demonstrated that passive administration of anti-Nocardia antibody to normal or T-cell depleted mice previously infected with Nocardia enhanced the inflammatory reaction and increased the tendency to slough off the infected leg (the detrimental effect was slower in normal mice than in T-cell depleted mice). For a more detailed account of the findings of these studies see sections 1.7.3.1.3 and 1.7.3.1.5.

Animals infected with N.brasiliensis showed low antibody titres to Nocardia (Ximenez et al, 1980) although N.brasiliensis is a potent polyclonal B-cell activator and a mitogen that will activate B-cells independently of T-cells (Ortiz-Ortiz et al,

1979). To explain this apparent incongruity, Conde et al (1983) suggested that antibody may be produced at the site of infection but concomitantly absorbed. They showed that anti-N.brasiliensis antibody and complement were present in the mycetoma lesion at a time when this antibody was absent from the serum, and it appeared that the immunoglobulin was produced in part locally and was specifically deposited at the site in the form of insoluble immune complexes, which could contribute to the pathogenesis of infection by impairing T-cell function and cell-mediated immunity (Conde et al, 1983).

1.8 CONCLUSIONS

Pathogenicity of Nocardia organisms is determined by a complex and multiple series of factors and varying combinations of these factors associated with the organisms will give rise to varying degrees of virulence and will dramatically influence the ability of the organism to establish itself in host tissue (Beaman et al, 1985). Various unique properties such as presence or absence of superoxide dismutase, catalase, resistance to phagocytosis, ability to inhibit phagosome-lysozome fusion or modulate lysozomal enzyme levels, ability to induce alterations in intraphagosomal pH, structure and composition of the cell wall, phase of growth all contribute to determine the pathogenicity of the organism (Beaman et al, 1985; Beaman and Black, 1985; Beaman et al, 1983; Beaman and Maslan, 1978; Black et al, 1983; Black et al, 1985; Davis-Scibienski and Beaman, 1980a and b; Vistica and Beaman, 1983; Beaman, 1979). The ability of the virulent strains of Nocardia to exist as facultative

intracellular parasites (Bourgeois and Beaman, 1974) within macrophages, protected from lysis (Beaman, Bourgeois and Moring, 1981) probably as L-forms is a strong pathogenic advantage, since any subsequent deficiency in host defence will favour development of active infection (Beaman, 1976 : 397; Beaman, 1980; Beaman and Scates, 1981).

Similarly, host defence consists of a complex combination involving both naturally present immune mechanisms and specifically induced immunological functions which act in concert in response to the infection. Cell-mediated immunity, activated macrophages and T-lymphocytes (involved both in activation of macrophages and capable of direct T-lymphocyte-mediated cytotoxicity to Nocardia) are important in host resistance (Beaman and Sugar, 1983; Krick and Remington, 1975; Deem et al, 1982; Deem, Doughty and Beaman, 1983; Beaman, 1977; 1979; Melendro et al, 1978; Ximenez et al, 1980; Folb, Timme and Horowitz, 1977; Sundararaj and Agarwal, 1977a; Filice, Beaman and Remington, 1980; Davis- Scibienski and Beaman, 1980c; Beaman, Gershwin and Maslan, 1978; Beaman, Gershwin et al, 1980b; Beaman, Goldstein et al, 1978). In addition, the inhibitory effects of PMN may be important (Filice, 1985).

The host-parasite interaction may therefore be compared to a delicately - balanced scale - any factor leading to deficiency in host immune function or which enhances the pathogenicity of the Nocardia organism may tip the scale in favour of progressive disease.

1.9 OBJECTIVES OF THIS STUDY

Pathogenic Nocardia are facultative intracellular pathogens (Bourgeois and Beaman, 1974) that reside and grow within macrophages (Beaman and Sugar, 1983). Host resistance is generally believed to depend upon successful collaboration between activated macrophages and specifically sensitized T-lymphocytes, in addition cell-mediated immunity, the inhibitory effects of PMN and direct T-lymphocyte-mediated cytoxicity are important (Beaman, Gershwin and Maslan, 1978; Beaman, Gershwin et al, 1980; Beaman, Goldstein et al, 1978; Beaman and Scates, 1981; Krick and Remington, 1975; Filice, Beaman and Remington, 1980; Beaman and Sugar, 1983; Deem, Beaman and Gershwin, 1982; Davis-Scibienski and Beaman, 1980c; Filice, 1985; Deem, Doughty and Beaman, 1982).

The macrophage is a major factor in the control of intracellular parasites (Rojas-Espinosa et al, 1982). Further, it has been established that initial interactions between Nocardia and macrophages are important in determining the extent of infection that develops (Beaman and Smathers, 1976; Beaman, 1977; Beaman, Goldstein et al, 1978; Black et al, 1983, 1985). Previous reports have suggested that both non-specifically activated macrophages (Filice, Beaman and Remington, 1980; Krick and Remington, 1975) and specifically activated macrophages (Beaman, 1979) are important in host defence against Nocardia (Davis-Scibienski and Beaman, 1980c). Although macrophages have been shown to be activated in N.asteroides infections (Davis-Scibienski and Beaman, 1980c; Sundararaj and Agarwal, 1977a,

1978; Krick and Remington, 1975; Beaman, 1979) and in N.brasiliensis infections (Melendro et al, 1978) it has been suggested that a deficiency or alteration in macrophage function occurs in association with the disease or as a result of host corticosteroid treatment (Beaman, 1976 : 394). This idea has also been expressed by Curry (1980) and Rojas-Espinosa et al (1982). On the basis of this consideration, a study of macrophage function in experimental N.asteroides and N.brasiliensis infections was undertaken. The study has attempted to determine whether certain biochemical defects or alterations in macrophage function occur in association with the disease. The concept of macrophage inactivation and thus the persistance of Nocardia has been investigated by others (Davis-Scibienski and Beaman, 1980a, 1980b; Black et al, 1983, 1985; Beaman and Black, 1985) and these workers have elucidated three of the mechanisms whereby virulent strains of N.asteroides appear to survive within macrophages, these being: the ability of the organism to inhibit phagosome-lysozome fusion, to induce alterations in intraphagosomal pH and to alter the activities of lysozomal enzymes (Beaman et al, 1985).

This investigation also attempts to directly examine the effects of Nocardia infection on macrophage function. Other studies have investigated whether macrophages are activated in Nocardia infections after inoculation with live organisms (Krick and Remington, 1975; Sundararaj and Agarwal, 1977a, 1978; Melendro et al, 1978, Filice, Beaman and Remington, 1980) and killed organisms (Davis-Scibienski and Beaman, 1980c) and have assessed

macrophage activation in terms of microbicidal activity (Davis-Scibienski and Beaman, 1980c; Sundararaj and Agarwal, 1977a, 1978; Melendro et al, 1978; Filice, Beaman an Remington, 1980) or in terms of tumour cell cytoxicity (Krick and Remington, 1975). Although the parameter of antimicrobial activity is accepted as the most definitive measure of macrophage activation (Karnovsky and Lazdins, 1978; North, 1978; Cohn, 1978) and stimulation of tumoricidal activity is also a lymphokine-mediated event (Cohn, 1978), biochemical parameters of macrophage activation in Nocardia infections with the exception of the recent studies of the lysozomal acid hydrolase, acid-phosphatase (Black et al, 1983, 1985) do not appear to have been reported in any detail. In addition, the assessment of macrophage activation is complicated by the use of sterile irritants (Melendro, et al, 1978) or adjuvants (Sundararaj and Agarwal, 1977a; Karnovsky and Lazdins, 1978). Further, the issue has been complicated by the finding the peritoneal macrophages from N.asteroides-infected mice were not activated, as assessed by microbicidal activity, in the study by Filice, Beaman and Remington (1980). Some other aspects of macrophage behaviour in experimental N.asteroides infection have been studied, eg macrophage migration inhibition (Sundararaj and Agarwal, 1977a), macrophage aggregation (Sundararaj and Agarwal, 1977a) and phagocytosis (Beaman, 1979; Davis-Scibienski and Beaman, 1980a and c; Black et al, 1983, 1985).

The study by Folb, Jaffe and Altmann (1976) led to the idea that macrophage function in N.asteroides and N.brasiliensis infections

may be different. This investigation corroborated the study by Uesaka et al, 1971; by showing that inoculation of N.asteroides and N.brasiliensis into experimental animals produced two different histopathological lesions. Lesions of N.asteroides were characterized by an acute, suppurative abscess, whereas in N.brasiliensis infections a granuloma was produced in which a striking feature was the presence of large numbers of foam-laden macrophages with intracellular organisms. The features of the 'brasiliensis lesion' (Uesaka et al, 1971) were reminiscent of the histopathological features of lepromatous leprosy and of disseminated Mycobacterium bovis infection when this occurs in the immune-suppressed situation. It was suggested that N.brasiliensis infection produces a depression of cellular immunity that modifies local host response to the organism (Folb, Jaffe and Altmann, 1976). This study investigates the possibility that macrophage responses in the two infections may be different by comparing biochemical and morphological parameters of macrophage function. On the basis of the histopathological studies by Folb, Jaffe and Altmann (1976) it is suggested that parameters of macrophage function may be depressed in N.brasiliensis infection. Alternatively, the degree of activation or inhibition of macrophage function may be different in N.asteroides and N.brasiliensis infections.

As already stated, initial interactions between Nocardia and macrophages play a significant role in the pathogenesis of Nocardia infections. Macrophage function was therefore investigated at 2, 7, 13 and 21 days post-inoculation. In

addition, such a study would hopefully determine whether macrophage responses to the two infections are modulated at these specific intervals over the 21 day post-inoculation period.

This study therefore, attempts to determine whether macrophage function is deficient in N.asteroides or N.brasiliensis infections; to directly examine the effects of Nocardia infection on macrophage function; to determine whether macrophage responses in the two infections are different; to assess macrophage responses early in infection and to investigate whether macrophage function is modulated over the 21 day post-inoculation period. Biochemical parameters of macrophage function selected are release of the enzymes plasminogen activator and lysozyme, in addition morphologic characteristics were investigated.

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CHAPTER 2

AN EXPERIMENTAL MODEL OF NOCARDIA INFECTIONS

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Chapter 2

AN EXPERIMENTAL MODEL OF NOCARDIA INFECTIONS

2.1 INTRODUCTION

This section describes the methods in general use in this study, the preparation of reagents, and discusses the Nocardia animal model and the culture of mouse peritoneal macrophages. Certain terms in general use in this dissertation are defined, or abbreviations given, and important concepts are discussed.

2.2 PREPARATION OF REAGENTS

2.2.1 TISSUE CULTURE MEDIUM

Dulbecco's modified eagle medium (DB). Gibco H-18 (Grand Island Biological Co., Grand Island, N.Y.) 3.7g sodium bicarbonate added per litre.

Hanks balanced salt solution (HBSS) Gibco K12. No sodium bicarbonate added. The media were sterilized by filtration through Millipore filters (pore diameter Ø.45um type HAWP, Millipore Company, Bedford, Mass. Ø173Ø USA).

2.2.2 SERUM

Foetal Calf Serum (FCS)

Gibco 22Ø-629Ø virus and mycoplasma screened. The serum was aliquoted aseptically into 1Ø ml aliquotes and stored at -2Ø°C.

Heat Inactivated Fetal Calf Serum (HIFCS)

Gibco 200-6290. The serum was heated at 56°C in a water bath for 30 minutes to destroy complement and then aliquoted aseptically into 10 ml aliquots and stored at -20°C.

Acid-Treated Dog Serum

Gibco 200-2500. The serum was adjusted to pH 3.2 by addition of 1N hydrochloric acid, incubated at room temperature for two hours and then neutralized with 1N sodium hydroxide. This acid treatment destroys the serum plasmin inhibitors (Unkeless, Gordon and Reich, 1974) - it destroys alpha $_2$ macroglobulin, an inhibitor of proteinase activity (Gordon, Werb and Cohn, 1976 : 344).

2.2.3 ANTIBIOTICS

Penicillin and Streptomycin (P+S)

Novo Strep: 1g streptomycin sulphate as base, manufactured by Novo Industries, Johannesburg, South Africa. 5 ml sterile normal saline added.

Crystapen 600 mg, containing 600 mg ($1x10^6$U) sodium benzylpenicillin. 5 ml sterile normal saline added.

5 ml penicillin and 5 ml streptomycin combined and diluted aseptically to 100 ml with sterile normal saline, and aliquoted into 1 ml aliquots and stored at -20°C. Each vial thawed once only and discarded.

Penicillin (100 IU/ml) and streptomycin (0.1 mg/ml) (Schnyder and Baggiolini, 1978a) was added to the tissue culture medium immediately prior to cell culture.

Mycostatin (M)

Squibb Laboratories (Pty) Ltd, Isando, Transvaal, South Africa. Contains Nystatin 100 000 u/ml.

30 units /ml Mycostatin (Schnyder and Baggiolini, 1978a) was added to the culture medium destined for macrophages to be assayed for lysozyme release and for indirect plasminogen activator (PA) assay. The medium was sterilized by filtration.

2.2.4 GENERAL REAGENTS

Phosphate Buffered Saline (PBS)

PBS 0.154 M pH 7.2

Recipe : NaCl 40 g

KCl 1 g

Na_2HPO_4 $2H_2O$ 7.21 g

K_2HPO_4 1 g

H_2O distilled to 1 litre = 5 times concentrated PBS

The salts were dissolved and the solution made up to volume.

Tris (hydroxymethyl) aminomethane Merck 8382 (TRIS-HCl)

0.1 M pH 8.1

The salt was dissolved in about half the required volume of distilled water. The pH was adjusted to 8.1 with 3N HCl and the

solution made up to volume and stored at 4^{o}.

Triton X-1ØØ

Merck Ant 11869 for scintillation grade. sg = 1.Ø5 g

Ø.5% V/V required i.e. Ø.5 ml/1ØØ ml distilled water

Ø.83% ammonium chloride (NH_4CL) Merck pH7.4

2.3 FLUORIMETRIC DETERMINATION OF TOTAL PROTEIN

2.3.1 PRINCIPLE

Fluram^{R} (fluorescamine) [4-phenylspiro {furan-2 (3),1 -phthalan} -3,3-dione] reacts with primary amines to form intensely fluorescent substances, providing the basis for a rapid and sensitive assay of amino acids, peptides, proteins and other primary amines (Udenfried et al, 1972). Fluram is non-fluorescent and any excess reagent is rapidly hydrolysed to form water soluble, non-fluorescent products.

The intensity of fluorescence is proportional to the amine concentation and the fluorophors are stable over several hours. At pH 8 to 9 the reaction occurs with a half-time of milliseconds, proceeding to near completion (approximately 8Ø-9Ø% of theoretical yield) even when Fluram^{R} is not present in large excess.

2.3.2 METHOD

Protein was determined spectrofluorimetrically (Hoal, 1981) using bovine serum albumin (BSA) as a standard. (Miles Scientific, Div of Miles Labs Inc, Naperville, Illinois 6Ø566, USA).

2.3.2.1 REAGENTS

Borate Buffer (Ø.2 M pH 9.2) (Merck)

6.18 g Boric acid was dissolved in 5ØØ ml of triple distilled water. The pH was adjusted to 9.2 with 2N sodium hydroxide.

Fluram^{R} (Hoffman-La Roche, Basle, Switzerland) was dissolved in acetone to give a final concentration of Ø.25 mg/ml.

Bovine serum albumin (BSA) was dissolved in borate buffer at a concentration of 1 mg/ml. 1 ml aliquots were stored at -2Ø^{o}C.

2.3.2.2 PREPARATION OF THE STANDARD CURVE

BSA 1 mg/ml in borate buffer was diluted 1:1Ø in borate buffer (= reagent A). The standard curve was prepared over the range 1-15 µg/ml and 12 standards were prepared 1-1Ø µg, 12 µg and 15 µg). The appropriate volume of reagent A for the required BSA concentration was made up to 8ØØµl with borate buffer. 2ØØµl of Fluram^{R} in acetone was added to the borate/BSA while vortexing vigorously to ensure complete mixing of the Fluram^{R} with the BSA solution.

The blank prepared was 25 µl of Ø.5% Triton X-1ØØ Ø.5% V/V added to 775 µl of borate buffer and combined with 2ØØ µl Fluram^{R}.

Sample volume was adjusted so that fluorimetric values fell well within the range of the standard curve. 25 µl sample was found

to be a suitable volume, and was added to 775 µl borate buffer and combined with 200 µl Fluram while vortexing very well.

Cell lysates in 0.5% Triton X-100 were vortexed vigorously to obtain a homogeneous suspension.

Samples, standards and blank were all prepared in duplicate in Corning light wall rimless pyrex glass tubes.

Fluorescence of standards, blank and samples was read on a Perkin Elmer MPF-44A fluorescence spectrophotometer (Perkin Elmer Ltd, Buckinghamshire, England HP9 1QA) with the excitation wavelength set at 390 nm (slit width 4 nm) and the emission wavelength set at 475 nm (slit width 6 nm).

2.3.2.3 CALCULATION OF RESULTS

A linear regression for recovery of x from y was run on the peak heights from the standards (Fig 1) and the unknown concentrations of the samples were calculated by interpolation.

2.3.2.4 SIGNIFICANCE OF RESULTS

Fluorimetric determinations of total protein were done on monolayer cultures of macrophages in order to determine specific activities of the enzymes studied and to gain an indication of cell number. The cells are washed twice with PBS after collection of the conditioned medium, as detailed in Chapters 3 and 4, and 1000ml 0.5% V/V Triton X-100 added to lyse the cells. The cell lysate is analyzed as detailed above for protein

content. Results obtained in this way permit calculation of total protein in the tissue culture dish. Specific activities of enzymes within cells and in the cell culture media may be related either to actual cell numbers or to the amount of cellular protein (Schnyder and Baggiolini, 1978a). Any non-adherent cells will be removed in the washing procedure, as will serum or other proteins in the culture medium.

2.4 NOCARDIA ORGANISMS

2.4.1 SOURCE OF NOCARDIA ASTEROIDES AND NOCARDIA BRASILIENSIS AND THEIR VERIFICATION

Nocardia asteroides, strain D1Ø39 was isolated from a patient, and obtained from Dr CN Young, South African Institute of Medical Research, Johannesburg, South Africa.

Nocardia brasiliensis was isolated from a subcutaneous abscess of a patient suffering from clinical illness (Folb, Jaffe and Altmann, 1976). The N.brasiliensis culture was confirmed by L Kaufman, Centre for Disease Control, Atlanta, Ga.

2.4.2 DETAILS OF CULTURE OF ORGANISMS, CONFIRMATION THAT INOCULATION IS A PURE GROWTH, HARVESTING OF ORGANISMS

Both N.asteroides and N.brasiliensis were cultured for 4 days on ISO sensitest agar plates (IST) OXOID CM471 at 3Ø^{o}C (Oxoid Ltd, Basingstoke, Hants RG24 OPW). Growth on agar plates gives rise to colonies of organisms which are at different stages of growth i.e. lag phase at the periphery, progressing through log and early stationary phase to stationary phase at the centre of the

colony.

Criticism has been levelled at the use of colonies of Nocardia grown on media and the inoculation of "crude" suspensions of organisms in the investigation of nocardial pathogenesis (Beaman and Maslan, 1978; Beaman et al, 1980; Beaman and Sugar 1983), since this method contributed to the contradictory reports concerning the virulence of Nocardia for mice (Uesaka et al, 1971; Gonzalez-Ochoa, 1973). The fact that other investigators did not standardize their techniques of growing, preparing and inoculating the organisms into the host has also been criticized (Beaman, Maslan et al, 1980). Consequently it has not generally been possible to compare the results of one study with those of another (Beaman, Maslan et al, 1980). In this study, virulence of Nocardia is not being investigated - virulence of the organisms was only a consideration in developing a reproducible non-lethal infection of similar degree with both N.asteroides and N.brasiliensis. In a previous study, Folb, Jaffe and Altmann (1976) found that N.asteroides and N.brasiliensis cause lesions in the experimental mouse that are quite different in their features (Section 1.7.3.1.1). This finding, which will be discussed in more detail at a later stage, gave rise to the idea that macrophage function in N.asteroides and N.brasiliensis infection in the experimental mouse may be different, and has been investigated in this study. The method of preparation of the inoculum (Folb, Jaffe and Altmann, 1976) was used since it had been employed in the original work leading to this investigation.

2.4.3 CONFIRMATION THAT INOCULUM IS A PURE GROWTH, AND HARVESTING OF ORGANISMS

The purity of the plates was checked visually with a 10x magnifying glass, and the colonies of organisms were harvested aseptically from the agar by scraping with a flamed glass slide, and placed in a pre-weighed sterile bottle. Great care was taken to remove as little agar as possible. Grams stains were done on single colonies for each harvest to further ensure purity of the culture and to confirm morphologically that the organisms were nocardiae. The organisms were suspended in sterile normal saline with the aid of a magnetic stirrer at the following concentrations:

N.asteroides 10 mg/ml wet weight

N.brasiliensis 20 mg/ml wet weight

The suspension was stirred vigorously for 30 minutes before inoculation of animals. Saline has been used as a medium for suspension of Nocardia by other workers (Krick and Remington, 1975: Uesaka et al, 1971; Beaman, Maslan et al, 1980).

2.4.4 ROUTE OF INOCULATION

Folb, Jaffe and Altmann (1976), Folb, Timme and Horowitz (1977), inoculated Swiss white mice intraperitoneally (i.p) with a suspension of live organisms. The method employed by these workers was followed exactly in this study. The mouse abdomen was swabbed with an alcohol swab (The Kendall Company, South Africa, isopropyl alcohol 70% V/V). After the animal inoculation procedure, an aliquot of the inoculum was cultured on ISO

sensitest (IST) agar plates to determine whether any contamination of the inoculum had occurred while the animals were being injected. Suspensions of the organism were introduced through a 26 gauge needle through the lower left abdominal wall. The needle was tilted slightly upwards in an attempt to avoid the organs of the animal. As the needle was removed the abdomen and site of inoculation were swabbed again.

Animals inoculated intraperitoneally with Ø.5 ml sterile normal saline were termed control, uninfected animals. Others have inoculated control mice with PBS (Deem, Doughty and Beaman, 1983).

Other workers have used the i.p. route for inoculation of animals with Nocardia organisms (Krick and Remington, 1975; Sundararaj and Agarwal, 1978; Wallace et al, 1979: Beaman, Maslan et al, 198Ø; Beaman and Scates, 1981). Infections induced by this route were chronic and extensive, characterized by slowly progressive multiple lesions throughout the peritoneal cavity (Beaman, Maslan et al, 198Ø). Wallace et al (1979) produced a chronic infection model using this route of inoculation in which large abscesses developed but only a 5%-1Ø% mortality occurred.

2.4.5 ESTABLISHMENT OF DOSE REQUIRED FOR SUB-LETHAL INFECTION

The dose of N.brasiliensis required to produce a sub-lethal Nocardia infection in mice was established at 1Ø mg wet weight suspended in Ø.5 ml normal saline per animal. Folb, Jaffe and Altmann (1976) used 5 mg dry weight per animal. The dose

selected produced a sub-lethal general peritonitis which was similar to that produced in Swiss white mice by Folb, Jaffe and Altmann, (1976). It was therefore felt to be unnecessary to use other doses of N.brasiliensis in this study.

N.asteroides D1Ø39 at a dose of 2Ø mg/ml wet weight caused a 5Ø% mortality in the mice inoculated. The dose of inoculum was reduced to 1Ø mg/ml wet weight, i.e. 5 mg wet weight was inoculated into each mouse. This produced a 5-1Ø% mortality and in many mice produced massive peritoneal infection and animals appeared very ill. In order to reduce the mortality and develop a more standardized peritoneal infection Ø.3 ml of the 1Ø mg/ml wet weight N.asteroides was inoculated i.e. 3 mg wet weight per animal. This gave rise to a reproducible sub-lethal general peritonitis. There were only occasional fatalities with these doses of organisms.

2.5 THE NOCARDIA ANIMAL MODEL

2.5.1 MOUSE STRAIN

We have used specific pathogen-free (Ha) ICR/UCT male mice, outbred, 25-3Ø g in weight in this study. In initial experiments performed when setting up the experimental system (3.3.1.5.1), several inbred strains of mice were used, these being BALB/c,/UCT, C_3H/He/UCT and CBA/Ca/UCT, all specific pathogen-free, male and 25-3Ø g in weight. (Ha) ICR/UCT mice were selected for this study on the basis of the results of these initial experiments (3.3.1.5.2) and because Folb, Jaffe and Altmann (1976) used outbred Swiss white mice (unobtainable for

this study) in the study which led up to this work. Also, cells from outbred mice give better cultures than those from highly inbred strains (Stuart, Habeshaw and Davidson, 1978 : 31.4).

2.5.2 MAINTENANCE OF ANIMALS

Mice were house in the Infectious Isolation Unit, Animal House, Medical School, University of Cape Town. Animals were kept six mice per cage, fed on water and rat cubes ad libitum and control mice were kept under the same conditions for the same period of time. Mice were monitored daily, and any dead mice removed immediately.

2.5.3 PASSAGING OF NOCARDIA AND FULFILLMENT OF KOCH'S POSTULATES

Nocardia organisms were cultured on IST agar plates, harvested and inoculated into mice, as detailed previously. Post-mortems were performed after peritoneal cell harvest to ensure that controls were infection-free and that Nocardia-inoculated animals showed evidence of infection. Abscesses in 2 or 3 different animals were aseptically incised and pus swabs taken. These pus swabs were incubated for one week in Trypticase Soya Broth (TSB) and streaked on IST plates. The plates were incubated at 30°C for four days and the colonies of organisms harvested and inoculated into mice as described previously. At various intervals post-inoculation i.e. 2, 7, 13 and 21 days mice were sacrificed and post-mortems performed to confirm the presence of Nocardia infection. Abscesses were incised, pus swabs taken and these were incubated and cultured as described above. Plates

were examined visually for pure growth of Nocardia and purity of culture was confirmed by Gram's stain of single colonies.

In an attempt to maintain virulence of the N.asteroides and N.brasiliensis, organisms were passaged through mice at regular intervals, as described above. Nocardia organisms were generally cultured on agar plates for two successive passages and then passaged through the animals.

2.5.4 AUTOPSY ON MICE : PATHOLOGICAL VERIFICATION OF MODELS

Post-mortems were performed after peritoneal cell harvest on each sacrificed animal. Control animals were examined to ensure that they were free of infection and inoculated animals were checked for positive signs of Nocardia infection.

2.5.4.1 METHOD

Positive signs of infection were taken as splenomegaly with or without scattered abscesses in the peritoneal cavity. Presence of typical lesions without splenomegaly was also accepted as positive. Incision of the lesions in a representative number of animals and culture of pure colonies of Nocardia confirmed presence of infection. Absence of infection was determined by findings of normal spleen size and absence of any lesions.

After peritoneal cell harvest the abdominal wall of the animal was incised and reflected to permit examination of the peritoneal cavity. The abdominal viscera were examined carefully and, in the case of N.asteroides-inoculated animals, the thoracic viscera

were inspected. The viscera were examined *in situ* initially and then were reflected to display the kidneys, spleen, gut mesentery, diaphragm, base of the liver, urogenital system, inferior vena cava, iliac veins and arteries, etc. In the *N.asteroides*-inoculated mice the heart and lungs were examined.

2.5.4.2 EXTERNAL APPEARANCE OF ANIMALS

Inoculated animals were examined and compared to control animals immediately prior to sacrifice.

Mice inoculated 2 days previously with either *N.asteroides* or *N.brasiliensis* appeared to have lost weight in comparison to control animals and appeared unwell and were sometimes acutely ill with attendant symptoms such as sunken eyes, harsh staring coats, loss of mobility and seemed dehydrated (determined by pinching of skin at the nape of the neck).

Appearance of animals 7 days post-inoculation with *N.asteroides* or *N.brasiliensis* was similar to their appearance 2 days post-inoculation, however condition of the animals appeared to have deteriorated as compared to 2 days post inoculation. Apparently marked loss of weight was commonly noted and animals generally appeared debilitated.

13 days post-inoculation, animals appeared to have recovered to a certain extent in they did not have the debilitated appearance seen 7 days post-inoculation. Some loss of weight was noticeable but coat appearance had improved and animals seemed mobile

(judged from amount of fighting in the cage and by ability to evade capture). A few animals were seriously ill but this was the exception.

Mice generally seemed well 21 days post-inoculation with N.asteroides or N.brasiliensis, with a few exceptions, loss of weight was often not noticeable and behaviour was similar to that of control animals.

2.5.4.3 POST-MORTEM FINDINGS

Post-mortems were conducted on 815 control mice, 446 N.asteroides-inoculated and 433 N.brasiliensis-inoculated mice. Only visible lesions were documented.

No abscesses were found in any of the control animals. If splenomegaly was suspected, the animals were discarded. Post-mortem findings were correlated and tabulated for 297 N.asteroides-inoculated and 315 N.brasiliensis-inoculated mice. Abscesses and/or splenomegaly was found in each peritoneal cavity.

Grading of splenomegaly was based on spleen weight. In initial experiments, spleens were arbitrarily graded according to the size as being normal or enlarged over the range of mild splenomegaly (+), moderate (2+), marked (3+) very marked (4+) and gross (5+). These spleens were then removed, cleaned of mesentary and weighed. The spleen weights and corresponding size grading were used to estimate degree of splenomegaly in

subsequent experiments. Data for spleen weights from control mice are as follows : median value Ø.1274 g, 95% confidence limits, lower limit Ø.1Ø92 g, upper limit Ø.1521 g; data for spleen weights from infected mice are shown in Table 1, as well as graded estimates of splenomegaly for 612 Nocardia infected mice, data for hepatomegaly and a summary of data on occurrence of abscesses. The latter data are presented in more detail in section 2.5.4.3.1 - 2.5.4.3.8.

2.5.4.3.1 Two day N.asteroides infection (31 mice)

Abscesses and adhesions were present in all the peritoneal cavities examined. A characteristic of the abscesses were that they were "floating" i.e. were not fixed to the peritoneum. Abscesses were generally small and paler than noted at longer intervals after inoculation. Abscesses occurred most commonly under the liver, on the liver, diaphragm and on the stomach. No necrotic areas were found.

2.5.4.3.2 Two day N.brasiliensis infection (46 mice)

"Floating" abscesses were again noted. Adhesions were present in some animals and abscesses were not as prolific as in the 2 day N.asteroides animals. Abscesses were most commonly found under the liver, in the gut and gut mesentary, on the diaphragm and stomach. The occasional large vascularised abscess was found on the bladder, anterior abdominal wall at the site of injection and on the stomach (up to Ø.6 cm long). No necrotic areas were found.

2.5.4.3.3 Seven day N.asteroides infection (72 mice)

Marked peritoneal adhesions were generally noted and numerous abscesses were present. Gross abscesses were often found medial to the spleen and inferior to the stomach, also in the gut. Most commonly affected areas were the kidneys, under the liver, diaphragm, bladder and spleen. 9 kidneys, 1 liver had an area of necrosis, 5 necrotic spleens were found. Occasionally abscesses were found on the lungs, upper diaphragm surface and the thoracic walls. Intrahepatic, intrasplenic and intrarenal abscesses were common.

2.5.4.3.4 Seven day N.brasiliensis infection (116 mice)

Marked peritoneal adhesions and multiple abscesses were present. Abscesses were found most commonly under the liver, scattered in the gut and gut mesentary, diaphragm, spleen (occasional gross abscesses found here), anterior abdominal wall and on the liver. No necrotic areas were found. Some large abscesses were vascularized. Intrasplenic and intrahepatic abscesses occurred frequently.

2.5.4.3.5 Thirteen day N.asteroides infection (103 mice)

Marked peritoneal adhesions and prolific abscesses were scattered in the peritoneal cavity. Gross abscesses were frequently found medial to the spleen and inferior to the stomach. Areas most commonly affected were the spleen, anterior abdominal wall, kidneys, liver, gut and stomach. Penetration of the thoracic cavity and lungs occurred in the most debilitated animals and scattered abscesses were found. Intraperitoneal, intrasplenic

and intrahepatic abscesses occurred frequently. 5 necrotic kidneys, 5 livers had areas of necrosis and 6 necrotic spleens were found.

2.5.4.3.6 Thirteen day N.brasiliensis infection (1Ø3 mice)
Marked peritoneal adhesions and multiple abscesses were present. Abscesses were found predominantly on the gut and in the mesentary, on the posterior abdominal wall and sometimes extending into the retroperitoneal area, under the liver, diaphragm, medial to the spleen and inferior to the stomach, and on the spleen. Some large, well vascularized abscesses were found, as well as some intrahepatic and intrasplenic abscesses. 1 necrotic spleen was found.

2.5.4.3.7 Twenty-one day N.asteroides infection (68 mice)
Marked peritoneal adhesions and multiple abscesses were found scattered in the peritoneal cavity. Abscesses seemed smaller than those in the mice inoculated 13 days previously and were generally less numerous. Areas most commonly affected were the spleen, kidneys, the area medial to the spleen and inferior to the stomach and the liver. A few large, well vascularized abscesses were found, as well as frequent intrahepatic and some intrasplenic and intrarenal abscesses. Penetration of the diaphragm occurred in the most debilitated animals and abscesses were scattered over the thoracic walls and sometimes the lungs. 2 necrotic kidneys and 3 necrotic spleens were found.

2.5.4.3.8 Twenty-one day N.brasiliensis infection (94 mice)

Abscesses occurred most frequently under the liver, on the diaphragm, on the gut and gut mesentary and on the stomach. Adhesions and abscesses were scattered in the peritoneal cavity but often appeared to be healing. Areas of necrosis were rare, as were gross vascularized abscesses.

2.5.4.3.9 General observations

Distinct organ tropism was found for N.asteroides and N.brasiliensis. N.asteroides infected the kidneys far more frequently and more severely than N.brasiliensis. Of 297 mice infected with N.asteroides, 170 kidneys or 29% of kidneys had abscesses whereas 62 kidneys in 315 N.brasiliensis-inoculated mice were affected (10%). N.brasiliensis seemed to infect the gut and the gut mesentary more often than N.asteroides did. N.brasiliensis abscesses were generally larger than N.asteroides abscesses. The approximate size of abscesses produced in the gut by N.asteroides was about 1 mm diameter and by N.brasiliensis about 2.5 - 3 mm diameter. Large abscesses scattered through the gut appeared to be characteristic of N.brasiliensis. N.brasiliensis infected the gut in 185 out of 315 mice (59%) and N.asteroides infected the gut in 130 out of 297 mice (44%). The liver was more frequently infected in N.asteroides-inoculated mice (112/297, 38%) than in N.brasiliensis-inoculated mice (98/315, 31%).

Peritoneal fat was more often infected in N.brasilienis-inoculated mice (42/315, 13%) than in N.asteroides-inoculated

mice (30/297, 10%). Hepatomegaly was more frequent in N.asteroides-inoculated mice (181/297, 61%) than in N.brasiliensis-inoculated mice (142/315, 45%)

Some animals infected with N.asteroides and N.brasiliensis developed a spinning syndrome. A similar phenomenon was found to occur in mice infected with N.caviae (Beaman and Scates, 1981) and N.asteroides (Beaman and Sugar, 1983).

From the appearance of the animals and the post-mortem findings, it seems that the acute stage of infection with both N.asteroides and N.brasiliensis is 2-7 days post-inoculation with chronic infection developing at 7-13 days. Beaman and Scates (1981) have described similar findings in that mice inoculated i.v. with N.caviae were acutely ill at 48 hours post-infection.

2.6 NOCARDIA ANTIGENS

Culture filtrate antigens of Nocardia asteroides (confirmed by Ruth Gordon, Rutgers University, New Jersey) and Nocardia brasiliensis (confirmed by L Kaufman, Centre for Disease Control, Atlanta, Georgia) were kindly given to us by Professor G Baum and were prepared in the following way:

Asparagine broth was prepared by dissolving 7 g of L-asparagine in 300 ml of sterile distilled water, and the following reagents were dissolved individually in approximately 25 ml of sterile distilled water, and each solution was added in sequence to the asparagine solution:

7.0 g ammonium chloride

1.5 g magnesium sulphate. $7H_2O$

0.9 g sodium citrate

1.3 g potassium hydrophosphate

25 g glycerol and 10 g of glucose were added, and the volume was brought to 1 litre with distilled water and sterilized by autoclaving at 120°C for 20 minutes.

The Nocardia antigens were prepared by inoculating the asparagine broth with large fragments of a colony of N.asteroides and N.brasiliensis respectively, grown previously for approximately two weeks on Sabouraud dextrose agar slants. The colony fragments were floated on the broth surface, the flasks were cotton plugged and incubated undisturbed at 23°C. Time of incubation for N.asteroides was 7 months, and 10 months for N.brasiliensis. After the incubation period the culture fluid was decanted and collected. The pooled, unheated culture fluids were sterilized by successive passage through 0.45 µm and 0.22 µm filters. The sterile filtrate was divided into 500 ml aliquots and dialysed at 4°C for 48 hours against distilled water. The dialysed material was freeze dried in tared containers and reconstituted in pyrogen-free 0.9% sodium chloride to contain 10mg (dry weight) of non-dializable solids per ml. The concentrated culture filtrate antigen preparation was sterilized by filtration, dispensed into sterile vials and stored at -20°C. The antigen preparation was thawed, aliquoted into volumes of 35 µl in sterile plastic vials, stored at -20°C and thawed immediately prior to use.

2.7 INTRAPERITONEAL INJECTION OF THIOGLYCOLATE MEDIUM

Peritoneal macrophages were elicited by intraperitoneal injection of Ø.75 ml of sterile Brewers thioglycolate medium (Unkeless, Gordon and Reich, 1974; Gordon, 1978; 33.6), prepared and stored according to manufacturer's instructions. Peritoneal cells were harvested by the standard methods described under section 2.8, four days after injection. Macrophages elicited by intraperitoneal injection of certain irritants such as thioglycolate medium exhibit certain physiological manifestations of activation in comparison to cells obtained from the unstimulated peritoneal cavity (Bloom et al, 1976 : 2Ø).

2.8 MACROPHAGES

Mouse peritoneal cells were harvested according to the methods of Cohn and Benson (1964), Stuart, Habeshaw and Davidson (1978: 31.5) and Edelson and Cohn (1976 : 335).

2.8.1 TECHNIQUE

The individual mice were killed in turn immediately prior to cell harvest. The animal was anaesthetized with ether, and was killed by cervical dislocation. The mouse was then pinned with its extremeties extended to a board covered with alcohol-damped paper towel, and the fur sprayed well with 7Ø% alcohol. The skin was lifted upward and away from the anterior abdominal wall with forceps, and an incision of about 3 cm long made through the skin. Great care was taken not to penetrate the anterior abdominal wall. The skin was cut, reflected away and pinned

back. 4 ml Dulbeccos medium (DB) with penicillin, streptomycin and heparin (heparin sodium, 25.ØØØ IU/ml. Weddel Pharmaceuticals Ltd) was injected into the peritoneal cavity. DB contained 5ØIU heparin per ml (Bray et al, 1983). The injection was made along the mid-anterior line, taking care to avoid puncture of the gut. If this occurred the mouse was discarded. The injected fluid was then circulated by shaking and prodding the peritoneal cavity for about two minutes. Care was taken to avoid damage to the liver as this results in presence of blood in the peritoneal wash. The peritoneal wall was then tented with toothed forceps and a tiny incision made with sharp-pointed scissors. Care was taken to avoid spillage of the peritoneal wash. A preflamed sterile pasteur pipette was used to aspirate the injected fluid, and the fluid was placed aseptically in a numbered 15 ml conical tube (Falcon) and the tube kept on ice. About 3/4 of the fluid was usually recovered. A post-mortem was then performed on the animal to ensure absence of infection in controls and evidence of infection in Nocardia-inoculated animals. The process was repeated with each mouse and the aspirated fluid placed in separate tubes and kept on ice. The peritoneal fluid was aspirated within 5 minutes of injection in an attempt to avoid contamination with organisms from the gut (Stuart, Habeshaw and Davidson, 1978: 31.5).

If post-mortem examination revealed unusual characteristics, e.g. splenomegaly in controls, no evidence of infection in inoculated animals, the tube containing the peritoneal fluid from that animal was discarded.

Contamination of cultures with many red cells usually markedly reduces macrophage adherence to glass; of if they do adhere, they undergo degeneration (Stuart, Habeshaw and Davidson, 1978 : 31.9). If any red blood cells were present in the peritoneal fluid, the fluid was centrifuged for 5 minutes at a relative centrifugal force (RCF) of 574 in a Sorval RC 3-B refrigerated centrifuge (Du Pont Company, Instrument Product, Biomedica Division, Newtown, Conn Ø647Ø) to pellet the cells. Pelleted cells were then resuspended in 3 ml of Ø.83% ammonium chloride (NH_4Cl) pH 7.4 (Pantalone and Page, 1977; Filice, 1983), and placed on ice for 1Ø minutes. Cells were then pelleted again, washed once in DB + P&S and resuspended in fresh DB + P&S.

Peritoneal aspirates from control mice were pooled, as were those from Nocardia-inoculated mice. Peritoneal aspirates from batches of 4-12 mice were usually pooled. The pooled cells were pelleted by centrifugation at a maximum relative centrifugal force (RCF) of 574. RCF was determined by the formula:

$$RCF = 11.17 \ (r) \left[\frac{N}{1ØØØ} \right]^2$$

where, r = radius of the rotor in cm

N = revolutions per minute

The radius at the centre line of the bucket, r_{CL} = 26.2 cm

Therefore: The maximum RCF at the centre of the bucket at 14ØØ rpm = 574.

About 2/3 of the supernatant fluid was aspirated and the cell pellet was resuspended in the remaining supernatant. The cells were mixed by gentle agitation with a sterile pasteur pipette and a drop of cell suspension was removed and placed in a counting tube (Greiner, Germany). Differential cell counts were performed according to the method of Baker, Silverton and Luckock (1966 : 519) in a haemocytometer chamber (Spencer^{R} Bright Line Improved Neubauer, 1/10mm deep), using white cell diluting fluid and a white cell pipette at a dilution of 1 in 20 and mixed well by shaking for 3-4 minutes. About a quarter of the mixture was then discarded and the appropriate volume of cell suspension run into the counting chamber. Total and differential cell counts were done using a Leitz Laborlux 12 microscope with 16x eyepieces and a 32x long working distance objective.

The number of cells per ml of cell suspension was calculated using a standard technique (Baker, Silverton and Luckock, 1966 : 520).

The cell suspensions were diluted to a cell concentration of $1x10^{6}$ or $2x10^{6}$ macrophages/ml in DB supplemented with the appropriate concentrations of serum, 100 IU/ml penicillin and 0.1 mg/ml streptomycin. 1 ml of the cell suspension was placed as a bubble over the coverslips, which were held in a sterile petri dish (Falcon 3001; Falcon Plastics, Div Becton, Dickinson and Co., Cockeysville MD 21030 USA). After attachment (10 min at $37^{o}C$, 5% CO_2), the petri dish was flooded with medium and reincubated in a Forma Scientific CO_2 incubator.

2.8.2 IDENTIFICATION OF MACROPHAGES

Differential cell counts were routinely done as wet preparations in a haemocytometer (West et al, 1968; Cohn and Benson, 1964; Stuart, Habeshaw and Davidson, 1978 : 31.6) as described previously. A characteristic of the normal mouse peritoneal macrophage is a reniform or horseshoe-shaped nucleus (Fedorko and Hirsch, 1970) although the macrophage nucleus may vary in shape from horseshoe-shaped to fusiform (Lessin and Bessis, 1972 : 735). Other morphological criteria used to identify macrophages are their size and the ratio of the nucleus to cytoplasm (Rhodes et al, 1979). The nucleus to cytoplasm ratio is < 1 in the macrophage (van Furth, Hirsch and Fedorko, 1970) ie it occupies less than half of the total cell area (Hirsch and Fedorko, 1970 : 16). Characteristics of the small lymphocyte are scanty, almost empty cytoplasm and the nucleus is rounded or ovoid, generally with a little indentation on one of its sides, and is large relative to the cytoplasm (Ham 1969 : 273, Feldman, 1972 : 752). The nucleus of PMN is lobular and occupies less space than the cytoplasm (Ham 1969 : 265). Macrophages were distinguished as monocyte-like phagocytes with a reniform or horseshoe-shaped nucleus (Cohn and Benson, 1964) which generally occupied less than half the total cell area.

Differential cell counts performed as wet preparations in a haemocytometer were initially done by two independent observers. Once these two cell counts had become consistently similar, counts were done by one observer.

Accuracy in differentiating macrophages from other cell types present in the peritoneal wash was checked with air dried, methanol fixed preparations stained with May-Grunewald-Giemsa (Schnyder and Baggiolini, 1978a; Patterson and Youmans, 1970). Peritoneal cells were resuspended at a concentration of $1x10^5$ cells/ml, 0.3 ml of cell suspension placed in a Shandon Elliot cytocentrifuge (Shandon Southern Products Ltd., Cheshire, England; Davis-Scibienski and Beaman, 1980c) spun at 800 rpm for 5 min and stained in a Hemastainer auto slide stainer (Geometric Data, Wayne, Penn 19087 USA). Slides were fixed in methanol for 10 minutes, filtered May-Grunewald 10 minutes, Giemsa stain with buffer (pH 6.8, 150 ml of 0.2 M KH_2 PO_4 and 67 ml of 0.2N NaOH -> 5 l) for 15 mins, distilled water and buffer 20 seconds, finally buffer for 4 mins. Slides were examined under oil by light microscopy using a 40x objective.

Phase contrast or light microscopic examination of a stained smear is the simplest and usually the most reliable method of differentiating lymphocytes, macrophages and PMN, although sometimes it is impossible to differentiate monocytic cells from large lymphocytes (Bloom et al, 1976 : 20).

Identification of stained cells was based on Diggs, Sturm and Bell (1970), and on Sanderson and Phillips (1981).

2.8.3 VIABILITY OF MACROPHAGES

Viability of harvested macrophages was determined prior to culture by Trypan blue exclusion (2% Trypan blue diluted to 0.2%,

mixed with an equal volume of cell suspension and examined microscopically).

Uptake of dye by the cells indicates non-viability. 100 cells were counted in each determination. Cell viability should be greater than 95% and in most cases it was. Median cell viabilities and their 95% confidence limits (Hollander and Wolfe, 1973) were calculated for control macrophages and Nocardia -activated macrophages at each interval post-inoculation studied. Median viabilities for control macrophages was greater than 95%; for N.asteroides-activated macrophages was equal to 98% in each case; and for N.brasiliensis-activated macrophages greater than 95% (Table 2).

2.8.4 PREPARATION OF COVERSLIPS

Glass coverslips (22 x 22 mm, Chance Propper Ltd., Smethwick, Warley, England) were boiled in soap solution for two hours, rinsed 20 times with triple distilled water, oven dried and sterilized by autoclaving (138^{o}C, 15 p.s.i., 30 minutes; Einstein, Schneeberger and Colten, 1976). Coverslips were distributed aseptically in tissue culture dishes immediately prior to seeding of cells.

2.9 INFLUENCE OF TIME COURSE OF INFECTION ON TOTAL NUMBER OF CELLS AND ON NUMBER OF PERITONEAL MACROPHAGES HARVESTED

The average number of peritoneal cells yielded per mouse was calculated in each experiment, as was the average number of

macrophages yielded per mouse. The latter data were calculated from the differential cell counts. This was done at each interval post-inoculation studied. Median cell numbers, macrophage numbers and median viability values and their 95% confidence limits (Hollander and Wolfe, 1973) were calculated from these data and are shown in Table 2 and Figs 2 and 3.

Median total number of cells and number of macrophages harvested from control animals remained relatively constant over the 21 day period, whereas median cell number in both N.asteroides and N.brasiliensis-inoculated animals was increased 2 days post-inoculation, continued to rise and peaked at 7 days post-inoculation and thereafter declined towards control levels at 21 days (Fig 2 and 3). These differences were statistically significant at the Ø.Ø5 level at 2, 7, 13 and 21 days (N.asteroides) and at 2, 7 and 13 days (N.brasiliensis).

Similarly, median macrophage number peaked at 7 days post-inoculation and declined towards control levels at 21 days post-inoculation in both N.asteroides and N.brasiliensis-inoculated animals. These differences were significant at the Ø.Ø5 level at 7, 13 and 21 days (N.asteroides) and at 2,7 and 13 days (N.brasiliensis) when compared with control macrophage number. Control peritoneal cell yields consisted generally of about 55-6Ø% macrophages, about 35-4Ø% lymphocytes and 1-5% PMN. Peritoneal cells harvested from mice inoculated previously with N.asteroides and N.brasiliensis consisted of about 35% macrophages, 15% lymphocytes and 5Ø% PMN, at 2 days post-

inoculation; 40% macrophages, 20-25% lymphocytes and 35-40% PMN at 7 days post-inoculation; 50% macrophages, 20-30% lymphocytes and 20-30% PMN at 13 days post-inoculation; and 55% macrophages, 30-35% lymphocytes and 10-15% PMN at 21 days post-inoculation. (These data are approximations, refer to Table 2 for median macrophage percentages). The finding that PMN comprised a large proportion of the peritoneal exudate early in infection was expected, since it is well known that initial infection with Nocardia is a pyogenic one with a predominantly polymorphonuclear cell infiltrate (Filice et al, 1980; Bradsher, Monson and Steele,1982; Rico et al, 1982). Patterson and Youmans (1970) immunized mice with M.tuberculosis and differential counts from May-Grunewald-Giemsa stained smears showed 65% macrophages and 35% lymphocytes and PMN for normal mice and 30% macrophages and 70% lymphocytes and PMN for immunized mice.

2.10 STATISTICAL TREATMENT OF DATA

To examine the significance of each experimental factor discussed in this thesis, control, control + antigen, test and test + antigen samples were processed simultaneously, portions being withdrawn at three or four time intervals for analysis. For logistical reasons, all samples could not be processed at the same time and the data bank assembled to describe the behavior of each factor consisted of data from several experiments. The general format can be described in tabular from as follows :

Factor (eg. cumulative release of lysozyme into medium)

	sampling time	S1	S2	S3	S4
Expt 1	Control:	a	b	c	d
(performed	test:	e	f	g	h
on day A)	test + antigen	i	j	k	l
Expt 2	Control:	m	n	o	p
(performed	test:	q	r	s	t
on day B)	test + antigen	u	v	w	x

etc

where each letter a,b,c,....,x denotes a block of results determined by assay.

Visual examination of the data showed certain apparent trends:

1. a tendency towards increase of factor with time eg. a<b<c<d (usually);
2. a tendency for test results to be larger than their corresponding controls, eg. e and i>a, f and j>b and so on (usually).

This tendency was noted in the series of experiments measuring plasminogen activator release. In the series of experiments measuring lysozyme release there was a tendency for test results to be smaller than or equal to their corresponding controls, eg. e and i<a, f and j<b and so on (usually).

However, the number of samples possible for each experiment was limited so that the number of items in each block (a,b,c,d, ...,l,m,n,o,....,x) were too few for significance of the factor

to be established. it was clearly necessary to combine blocks from different experiments, thus:

Factor

sampling time	S1	S2	S3	S4
Control	A	B	C	D
test	E	F	G	H
test + antigen	I	J	K	L

where A is collected data from Expt 1(a) and Expt 2(m) and Expt 3 etc.

The magnitude of corresponding data from different experiments differed markedly (eg. a was appreciably different from m), as did the test/control ratios (e/a was appreciably different from q/m), so it was clear that the data had perforce to be treated as ordinal rather than interval in nature. Random tests also indicated that variances were not homogenous nor was the distribution of data within each block A,B,C,...L normal. The decision was therefore taken to use the non-parametric Mann-Whitney test (Siegel, 1956) to compare control and test groups; and medians and their 95% confidence limits were calculated by a method due to Tukey (Hollander and Wolfe, 1973) or due to Snedecor and Cochran (1980 : 473, Table A7). Where data was insufficient to calculate confidence limits using this method, 95% confidence limits of the median were calculated using the substitute t-test based on the range developed by Lord (1947; Snedecor and Cochran, 1980).

The following formula was used:

$((HR-LR) * tw)/_2 = X$

UL = M + X

LL = M - X

where: M = Median

HR = highest reading

LR = lowest reading

n = 2 tw = 6.353

= 3 tw = 1.304

= 4 tw = Ø.717

= 5 tw = Ø.5Ø7

= 6 tw = Ø.399

This test gives significance at the 5% level for a 2-tailed test.

2.11 INFLUENCE OF DURATION OF INCUBATION ON CELL PROTEIN (AS A MEASURE OF CELL NUMBER)

Schnyder and Baggiolini (1978a) studied macrophages from untreated and thioglycolate-treated mice, and found that cell numbers decreased with time in culture. Cell number, whether expressed as actual number of macrophages or as µg of cell protein dropped sharply in the first 2 days of incubation in the untreated cultures but cell loss subsided thereafter, whereas only slight cell loss was observed in thioglycolate-elicited macrophages. When mouse peritoneal macrophages are incubated in complete culture medium, a consistent loss of cells is seen with time (Lazdins et al, 1978). Patterson and Youmans (197Ø) found 1ØØ% cell survival over the first 5 days in culture, but thereafter cell numbers declined.

To determine whether cell number decreased with time in this experimental system, total cell protein was measured spectrofluorimetrically. The data in Tables 3-10 are the data used in Chapter 4 (4.2.4) experimental series "b" to determine lysozyme specific activities and the experimental methods used are described in this section.

In both types of culture, cell numbers remain similar or appear to decrease slightly with time of incubation, however there does not seem to be any clear trend, and in some cases cell protein is higher in final cultures than it was in initial cultures. This is thought not to be due to cell division as no dividing cells were ever seen in culture, but rather to be due to pipetting error when cells were seeded. Cells were, however, resuspended immediately prior to being seeded into culture dishes in an attempt to avoid such errors. In addition, these data are from replicate cultures (see section 4.2.4 for clarification), not from a single culture sampled over 4 days.

In some experiments it appears that cell loss in control cultures is higher than that of Nocardia-activated cultures during the first two days, in agreement with the literature.

From the data presented, it seems that decrease in cell number with time of incubation is not a significant factor in this study.

2.12 MORPHOLOGICAL EXAMINATION OF CULTURES

Macrophage cultures were subjected to thorough morphological examination immediately prior to washing and addition of fresh medium and before each sample was taken, using a Leitz Diavert phase-contrast inverted microscope. The criterion of cell morphology was used to determine viability of cultured macrophages. The morphological appearance of pseudopodia and ruffled membranes is indicative of cell viability (Patterson and Youmans, 1970), as is adherence of the macrophages to the substrate (Gangadharam and Pratt, 1983). Healthy culture have large, well spread cells firmly adherent to the bottom of the dish. Unhealthy cultures contain many refractile rounded cells that detach from the dish floor (Edelson and Cohn, 1976 : 337). Any cultures that appeared unhealthy were discarded.

2.13 THE CONCEPT OF THE ACTIVATED MACROPHAGE

In his review of the mononuclear phagocytic system, Lasser (1983) put forward the following definition: "activated macrophages can be defined as the morphologic, biochemical and functional changes that these cells undergo in expressing enhanced resistance to microorganisms, inflammation or neoplasia as compared with the baseline values of resident and stimulated macrophages".

Around the turn of the century, Metchnikoff wrote about his idea that macrophages needed to undergo functional changes to be able to show resistance to infection with certain bacteria (North, 1978).

Lurie's studies (1942) showed that macrophages in tuberculosis infection were altered and were able to reduce the growth of phagocytosed organisms, in contrast to normal macrophages.

In the 196Ø's Mackaness (1962; 1964; 1969) described the functional changes that occur in macrophages in animals infected with facultative intracellular bacterial parasites, and he introduced the term "activated macrophage" to describe the enhanced microbicidal activity of these cells. "Activation" has a specific immunologic basis, but its expression is non-specific. Mackaness showed that macrophages from immune animls had acquired the capacity to destroy intracellular bacteria, whereas normal macrophages were killed by unrestricted intracellular bacterial replication. Mackaness (1969) also elucidated the relationship between specific delayed-type hypersensitivity and the antimicrobial activity of macrophages. This work led to numerous studies into the morphological, biochemical and functional differences between these "activated" cells and normal cells. Almost 5Ø functional modifications have been shown to occur in activated macrophages (Lasser, 1983; Karnovsky and Lazdins, 1978; Cohn, 1978; Hopper, Wood and Nelson, 1979; Table 11).

The finding that macrophages with increased microbicidal activity also displayed certain biochemical or functional modifications gave rise to the idea that macrophages manifesting altered biochemical characteristics, were "activated". Evidence that macrophages elicited in the peritoneum by non-specific inflammatory agents such as thioglycolate medium, peptone and

sterile caseinate exhibited biochemical differences also manifested by "activated" macrophages led some workers to believe that macrophages obtained in this way are "activated". However, biochemical modifications produced by non-specific inflammatory stimuli do not imply that the macrophage has enhanced microbicidal activity. Karnovsky and Lazdins (1978) believe that the chemical and biochemical evidence available is insufficient to define non-immunologically elicited macrophages as "activated".

Cells from animals inoculated with agents such as thioglycolate have also been referred to as "stimulated" or "non-specifically activated". Other terms which have been used to subdivide macrophage activation into categories include "induced", "primed" and "armed" (Morahan, 1980). The lack of uniform terminology hampers studies on mononuclear phagocytes, as confusion results from terms being used interchangeably. Although some workers (Ogmundsdottir and Weir, 1980) restrict their use of the term to macrophage conforming to the original criterion as put forward by Mackaness, others such as North (1978), Cohn (1978) and Karnovsky and Lazdins (1978) feel that this is a much restricted definition. Cohn (1978) suggested that "properties of both qualitative and quantitative nature that exceed the baseline values exhibited by the resident unstimulated peritoneal macrophage should be considered as activated". However, differences exist in the state of activation shown by macrophages involved in a non-specific inflammatory response and those modified by lymphokines.

Karnovsky and Lazdins (1978) define "activated macrophages strictly as being those macrophages that have been activated by immunologic mechanisms in vivo". Listeria and BCG infections are given as examples of agents having given rise to activated macrophages. North (1978) suggests that workers describe macrophages as being activated metabolically, bactericidally, phagocytically etc. and that the way in which macrophage activation was induced be referred to in the same way.
The definition put forward by Lasser (1983) is a synopsis of the ideas of North (1978), Karnovsky and Lazdins (1978) and Cohn (1978) on this matter. The Reticuloendothelial Society (Morahan, 1980) has recommended that a qualified description be given in the form "macrophages from .....were obtained by ..... for.....function". The term "elicited" should be reserved for the experimental procedure that results in the accumulation of macrophages at a particular anatomical site. These recommendations have been followed in this dissertation. Accordingly, the system being used in this study is as follows: macrophages from mice were obtained by intraperitoneal inoculation of live organisms of N.asteroides and N.brasiliensis respectively, for secretion of plasminogen activator and lysozyme and for studies on morphology. In order to reduce repeated use of this definition in this text, this qualified description has been shortened to "Nocardia-activated macrophages".

"Control macrophages" are defined as those resident peritoneal macrophages harvested from specific pathogen free mice 2, 7, 13 and 21 days after inoculation with sterile, pyrogen-free isotonic

saline.

The presence of one manifestation of activation does not automatically indicate that others are present. Nogueira, Gordon and Cohn (1977) demonstrated that although the T-cell mediated increased capacity of mouse macrophages for destroying Trypanosoma cruzi may always be associated with increased secretion of plasminogen activator, the reverse need not be true. Activation is not necessarily an all-or-nothing phenomenon that yields a cell with increased ability to deal with all situations. It has been suggested by North (1978) and Cohn (1978) that the presence of one manifestation of macrophage activation in the absence of others may indicate that activation occurs in definite steps and that the number of activation steps is regulated by the homeostatic functions the macrophage is required to perform. Cohn (1978) has outlined the stages in the activation of mononuclear phagocytes. Non-specific inflammatory stimuli such as injection of thioglycolate medium induces a large number of the characteristics of activated macrophages, however a lymphokine-mediated event is required to induce microbicidal and tumoricidal activty. In this study, the definition of "activated" macrophages that is accepted is that of Karnovsky and Lazdins (1978) who defined "activated macrophages" as being "those macrophages that have been activated by immunologic means in vivo". This, in fact, concurs with Cohn's idea of a fully activated macrophage, and with Lasser's definition of activated macrophages.

There are numerous morphologic, biochemical and functional changes that are manifested in activated macrophages as compared to baseline values exhibited in unstimulated macrophages. The absolute relevance of several of the changes to the process of activation is debatable (Karnovsky and Lazdins, 1978). However, these changes characterise activated macrophages as compared to control macrophages. Some of these properties of activated macrophages were selected as parameters of macrophage function in Nocardia infections in mice. In this study, macrophage function was measured in terms of enzyme secretion, ie. plasminogen activator and lysozyme production: and morphological changes were assessed by phase contrast and scanning electron microscopy.

2.14 FIGURES

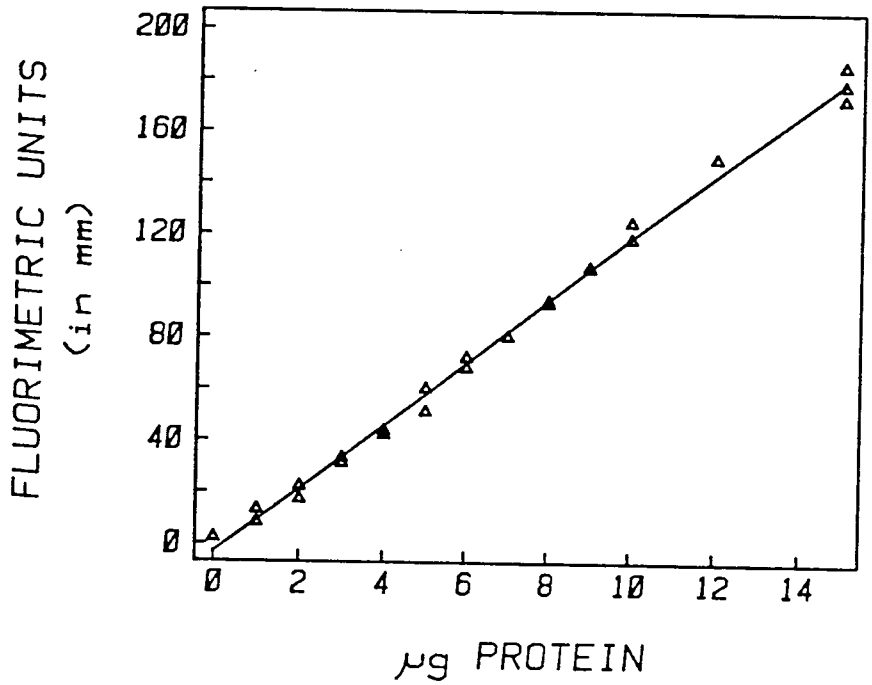

Fig 1: Linear regression for recovery of x from y

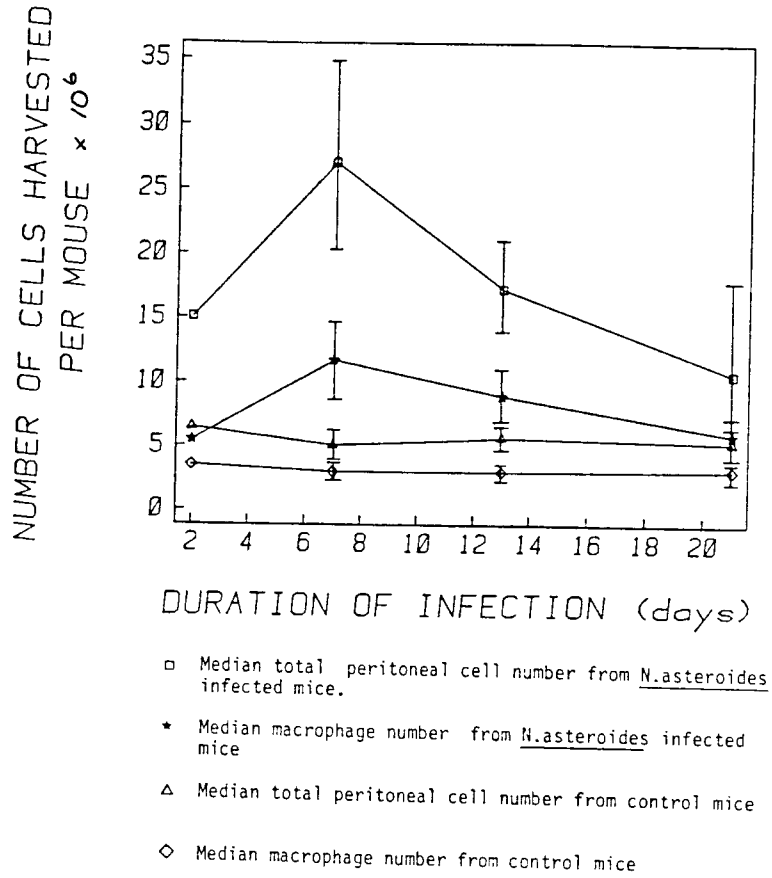

Fig 2 Influence of duration of N.asteroides infection on number of peritoneal cells harvested per mouse: Median values and their 95% confidence limits

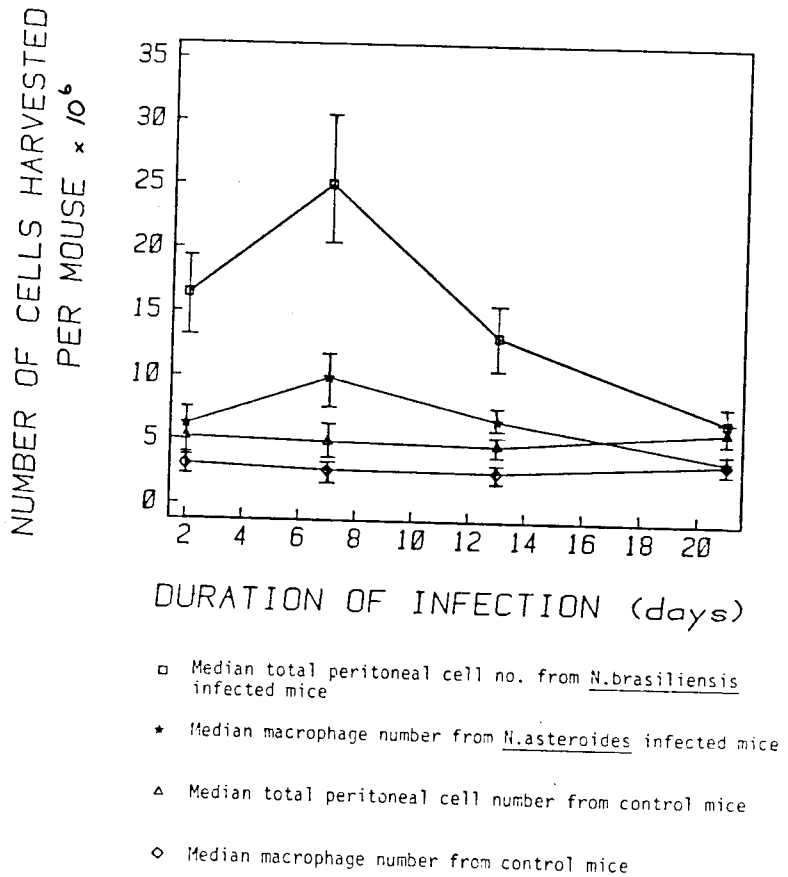

Fig 3 Influence of duration of N.brasiliensis infection on number of peritoneal cells harvested per mouse: Median values and their 95% confidence limits

2.16 TABLES

TABLE 1a

Summary of post-mortem findings

	No.	Splenomegaly						H
		None	1+	2+	3+	4+	5+	
*	42	M Ø.1274	Ø.1873	Ø.2992	Ø.5151	Ø.723	1.Ø14	
		LL Ø.1092	Ø.1748	Ø.2413	Ø.4545	Ø.6135	Ø.921	
		UL Ø.1521	Ø.204	Ø.3735	Ø.5661	Ø.802	1.165	
**		<Ø.16	Ø.17-Ø.23	Ø.24-Ø.43	Ø.44-Ø.58	Ø.59-Ø.85	>Ø.86	
2d Na	31	5	7	16	3			1Ø
2d Nb	46	7	24	14	1			11
7d Na	81	3	6	34	24	13	1	44
7d Nb	72		1Ø	47	12	3		38
13d Na	117	2	9	39	42	24	1	77
13d Nb	1Ø3	6	22	41	23	1Ø	1	55
21d Na	68	1	11	32	17	7		5Ø
21d Nb	94	13	41	28	11	1		36

KEY: No. = No. of mice
H = Hepatomegaly
* = Results from spleen grading experiment (weight in grams)
** = Arbitrary spleen weight range in grams
1+ = Mild splenomegaly
2+ = Moderate splenomegaly
3+ = Marked splenomegaly
4+ = Very marked splenomegaly
5+ = Gross splenomegaly

TABLE 1b

Summary of post-mortem findings

	Presence and site of abscesses
2d Na	Small, pale, "floating" abcesses, most commonly on and under liver, diaphragm and stomach.
2d Nb	Small, pale, "floating" abcesses scattered in peritoneal cavity, liver, gut, diaphragm and bladder.
7d Na	Marked peritoneal adhesions and numerous abscesses, especially on kidneys, liver, diaphragm and spleen.
7d Nb	Marked peritoneal adhesions and multiple abscesses, especially on gut, diaphragm, liver and spleen.
13d Na	Marked peritoneal adhesions and prolific abscesses, especially on spleen, kidneys, liver and stomach.
13d Nb	Marked peritoneal adhesions and multiple abscesses, especially on gut, liver, diaphragm and spleen.
21d Na	Adhesions and multiple abscesses scattered in peritoneum, especially spleen, kidneys and diaphragm.
21d Nb	Abscesses found most frequently under liver, on diaphragm, gut and stomach.

TABLE 2

Influence of time course of infection on total number of cells and on number of peritoneal macrophages harvested per mouse : Median values and their 95% confidence limits.

CELL TYPE			CONTROL				NOCARDIA-ACTIVATED			
a	b		c	d	e	f	c	d	e	f
2d Na	6	M	6.4	56	3.47	97.5	15.03	35.5	5.43	98
		LL	5.7	54	3.01	97.3	11.27	32.71	3.91	97.4
		UL	7.1	58	3.93	97.7	18.79	38.29	6.95	98.6
2d Nb	10	M	5.21	60	3.1	97.5	16.45	37	6.18	98.5
		LL	4.04	52	2.71	96	13.12	35	4.71	98
		UL	6.35	69	3.42	99	19.21	40	7.51	99.5
7d Na	15	M	5.0	58	2.94	98	27.03	43.5	11.67	98
		LL	4.11	55	2.47	97.5	20.3	40.5	8.74	97
		UL	5.93	60.5	3.48	99	34.39	46	14.61	98.5
7d Nb	14	M	4.94	55.5	2.71	96.5	25.04	39.5	9.96	96.5
		LL	3.83	53	2.09	94.5	20.51	37.5	8.04	93
		UL	6.43	58	3.76	98	30.33	42	11.86	98.5
13d Na	14	M	5.59	57	2.94	98	17.24	49.5	8.9	98
		LL	4.88	55	2.53	97.5	14.03	47	7.04	97
		UL	6.55	58	3.37	99	21.12	52	10.86	99
13d Nb	21	M	4.73	57.5	2.64	96	13.21	50	6.65	97
		LL	4.05	55	2.36	95.5	10.89	48	5.44	96
		UL	6.53	59.5	3.11	97	15.6	52	7.88	97.5
21d Na	11	M	5.25	56.75	3.09	97.5	10.58	56.75	5.87	98
		LL	4.54	50.5	2.57	97	7.4	52	4.26	97
		UL	6.27	59.5	3.63	98	17.82	63	10.62	98.5
21d Nb	15	M	6.01	59	3.51	96.75	6.77	53.25	3.64	96
		LL	5.46	54	3.19	95	5.79	49	3.08	94.5
		UL	6.79	62.5	3.95	97.5	8.29	56.5	4.14	98

KEY: a = Experimental type
b = Number of observations
c = Total cell number x 10^6
d = % Macrophages
e = Macrophage number x 10^6
f = % Viability

TABLE 3

2 day *N.asteroides* infection : Influence of duration of incubation on cell number (expressed in terms of protein content). Units = µg of cell protein.

	S1	S2	S3	S4
Control	215.8	313.92	269.16	209.8
		179.68	189.12	
	332.24	355.24	280.28	255.32
	462.16	335.24	325.24	274.28
Na		238.2	275.2	369.84
	276.92	243.36	214.08	455.92
	309.6	254.52	222.68	279.48
	247.32	277.28	266.28	624.04
	214.32	232.32	198.36	593.08
Na + 0.002% Ag	292.29	197.36	262.28	218.32
	200.36	316.28	264.28	531.12

TABLE 4

2 day *N.brasiliensis* infection : Influence of duration of incubation on cell number (expressed in terms of protein content). Units = µg of cell protein.

	S1	S2	S3	S4
Control		497.12	450.16	308.28
	615.04	568.08	402.2	289.28
	271.72	203.44	163.68	154.2
	281.24	254.44	156.8	208.64
	243.44	211.24	165.44	179.24
Nb	434.16	369.24	266.28	262.28
				288.28
	210.36	213.8	192.2	192.97
	129.12	193.96	285.56	130
	219.88	184.44	176.64	216.4
			183.56	182.72
Nb + 0.002% Ag	392.2	340.24	307.28	339.24
	244.08	248.40	180.12	187.88
	163.68	201.72	182.72.	151.6
	327.88	222.48	180.8	193.96

TABLE 5

2 day *N.asteroides* infection : Influence of duration of incubation on cell number (expressed in terms of protein content). Units = µg of cell protein.

	S1	S2	S3	S4
Control	267.68 239.32 491.2 547.4	261.8 241.76 346.04 320.12	234.84 75.6 225.04 255.28	446.64 414.28 237.28 256.88 296.12
Na	146.2 185.08 154.28 318.68 157.64 145.56 153.32 158.52	176.52 179.16 172.2 176.16 167.16 180.12 165.44 180.12	134.76 215.04 225.56 130.0 272.6 186.16 151.28 142.08	153.48 219.88 160.76 232.04 217.64 335.36 379.68 258.36
Na + 0.002% Ag	188.32 265.24 155.08 161.12 139.48 251.84 142.96	176.16 183.44 229.6 193.08 136.92 165.44 156.8	228.0 234.48 212.2 174.92 199.12 190.48 215.56	211.8 255.52 189.32 185.68 195.84 190.76

TABLE 6

7 day *N.brasiliensis* infection : Influence of duration of incubation on cell number (expressed in terms of protein content). Units = µg of cell protein.

	S1	S2	S3	S4
Control	236.56 207.0 234.84	276.6 281.8	287.92 237.44	262.68 213.96
Nb	167.84 190.44 178.28 188.72	287.72 203.52 225.24 170.44	155.64 257.44 241.8 186.12	191.32 153.04 174.8 186.12
Nb + 0.002% Ag	197.4 184.84 172.2 210.48	170.44 231.36 200.04 233.36	203.52 210.48 160.88	170.44 220.92 188.72 140.88

TABLE 7

13 day N.asteroides infection : Influence of duration of incubation on cell number (expressed in terms of protein content). Units = µg of cell protein.

	S1	S2	S3	S4
Control	187.44 151.68	245.92 183.36	161.4 212.6	185.8 224.8
Na	136.24 173.6	166.28 146.8	202.84 223.16	85.04 193.12
Na + 0.002% Ag	139.55 216.68	197.16 216.68	179.28 130.56	117.56

TABLE 8

13 day N.brasiliensis infection : Influence of duration of incubation on cell number (expressed in terms of protein content). Units = µg of cell protein.

	S1	S2	S3	S4
Control	416.56 371.04 587.2 385.68	267.88 342.6 323.92 310.6	299.44 285.64 306.36	195.68 181.84 296.88 220.76
Nb	240.24 219.92 198.0	214.24 235.36 270.32	200.0 195.68 161.96	218.16 209.52 173.2
Nb + 0.002% Ag	278.44 256.48	296.32 199.12 135.12	218.16 211.88	231.12 273.52 206.92

TABLE 9

21 day *N.asteroides* infection : Influence of duration of incubation on cell number (expressed in terms of protein content). Units = µg of cell protein.

	S1	S2	S3	S4
Control	415.36	398.92	209.52	141.2
	436.12	237.2	222.72	233.96
	317.2	325.92	495.24	242.64
	342.64	400.04	323.0	212.84
	304.94	264.88		
Na	287.36	257.08	226.8	76.32
	318.48	290.8	232.0	174.32
	284.76	262.28	230.72	130.84
	263.44	198.04	362.24	158.16
	270.68	201.68	145.0	159.4
	277.96	242.36	148.96	
	276.52	207.48		
Na + 0.002% Ag	244.12	321.08	172.32	140.32
	254.0	197.32	176.96	124.0
	232.92	222.72	111.56	122.76
	221.28	190.76	169.36	132.08
	227.08	201.68	140.0	119.68
				106.64

TABLE 10

21 day *N.brasiliensis* infection : Influence of duration of incubation on cell number (expressed in terms of protein content). Units = µg of cell protein.

	S1	S2	S3	S4
Control	296.68	255.04	169.96	210.96
	266.24	279.92	253.2	208.48
			215.32	260.64
Nb	367.28	393.36	502.64	479.04
	435.6	346.16	378.48	448.0
		333.36	325.04	329.92
Nb + 0.002% Ag	448.0	430.64	374.72	477.6
		342.4	405.76.	343.68

TABLE 11

SOME CHARACTERISTICS OF ACTIVATED MACROPHAGES *

MORPHOLOGIC - Increases in :

Size (increased protein content)
Adhesiveness and spreading
Ruffled membrane activity
Cytoplasmic granules

BIOCHEMICAL - Increases in :

A. **Metabolic activity**

Glucose oxidation
Lactic dehydrogenase
Lactate production
Oxygen uptake
Adenyl cyclase
Cyclic GMP
Ca^{2+} influx
ATP level
O_2 release
H_2O_2 release
Glucosamine uptake

B. **Secretions**

Plasminogen activator
Lysozyme
Collagenase
Elastase
Prostaglandins
Interferon

C. **Enzymes**

Lysozymal hydrolases and their release
Acid phosphatase
B-Glucuronidase
Cathepsin D

FUNCTIONAL - Increases in :

Pinocytosis
Phagocytosis (stimulation of E IgM C_3b ingestion)
Intracellular microbicidal activity
Cytotoxic effects on tumour cells

* Adapted from Lasser, 1983; Cohn, 1978; Karnovsky and Lazdins, 1978 and Hopper, Wood and Nelson, 1979.

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SECTION II

IN VITRO SECRETION OF LYTIC ENZYMES BY PERITONEAL MACROPHAGES FROM MICE PREVIOUSLY INOCULATED WITH PATHOGENIC NOCARDIAE

LYTIC ENZYMES IN CHRONIC INFLAMMATION

The secretory enzymes of macrophages are a major factor in many pathological processes (Baggiolini and Schnyder, 1982). These enzymes are able to degrade a great variety of biological molecules and this gives rise to two particularly important effects: the conditioning of the pericellular environment and tissue damage as a result of the breakdown of extracellular connective tissue structure. Modulation of rate of secretion of lytic enzymes is often used as a parameter of macrophage function. They are among the best defined truly secretory molecules of the macrophage and are very easy to assay (Baggiolini and Schnyder, 1982).

There are three classes of lytic enzymes secreted by macrophages: lysozyme, lysozomal hydrolases and neutral proteinases. These classes of enzymes are secreted according to different kinetics and their responses to agents which modify macrophage function are different. Among the three enzyme classes, lysozyme, beta-glucuronidase and N-acetyl-beta-glucosaminidasse (lysozomal hydrolases), and plasminogen activator (neutral proteinases) have been studied most frequently. The studies have shown that for all three classes of enzyme, secretion is a long lasting process which depends strictly on de novo synthesis (Gordon, Todd and Cohn, 1974); Schnyder and Baggiolini, 1978a and b).

The lytic enzymes selected in this study were lysozyme and plasminogen activator and macrophage function was measured in

terms of secretion of these enzymes.

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CHAPTER 3

NOCARDIA ASTEROIDES AND NOCARDIA BRASILIENSIS INFECTIONS IN THE MOUSE: INDUCTION OF PLASMINOGEN ACTIVATOR SECRETION BY MACROPHAGES

Chapter 3

NOCARDIA ASTEROIDES AND NOCARDIA BRASILIENSIS INFECTIONS IN THE MOUSE : INDUCTION OF PLASMINOGEN ACTIVATOR SECRETION BY MACROPHAGES

3.1 LITERATURE REVIEW

3.1.1 INTRODUCTION

Following the lead of Metchnikoff for the last 70 years, investigators of mononuclear phagocytes concentrated on studies of phagocytosis and intra-cellular digestion of particles (Nathan, Murray and Cohn, 1980). Recently, another role of mononuclear phagocytes has been recognized - their ability to synthesize and secrete a variety of biologically active products into their extracellular environment . The cells are capable of secreting a wide range of materials such as enzymes, complement components, enzyme inhibitors, endogenous pyrogen, oxygen metabolites, bioactive lipids, tumoricidal agents and growth promoting factors (Nathan, Murray and Cohn, 1980; Lasser, 1983; Hamilton and Moore, 1980).

Macrophages can secrete a variety of proteases active at neutral pH, such as plasminogen activator (Unkeless, Gordon and Reich 1974); collagenase (Wahl et al, 1974) and elastase (Werb and Gordon, 1975). A very large number of studies have shown beyond doubt that plasminogen activator and the other neutral proteases are secreted by macrophages that have been exposed to a stimulus and have adopted functionally to it, i.e. neutral

protease secretion is dependent on the physiological state of the cells and is a characteristic of elicited and activated macrophages (Baggiolini and Schnyder, 1982; Cohn, 1978; Vassali, 1980).

Plasminogen activator has been shown to be synthesized by a variety of cell types and it is clear that the enzyme is associated with many biological events in which controlled and localized proteolysis is involved, such as in embryo implantation, (Strickland, Reich and Sherman, 1976), follicle rupture at ovulation (Beers, Strickland and Reich, 1975), and post-lactational mammary involution (Ossowski, Biegel and Reich, 1979). Release of the enzyme has been shown to be a property of oncogenically transformed fibroblasts (Unkeless et al, 1973), activated macrophages (Unkeless, Gordon and Reich, 1974) macrophage cell lines (Hamilton and Moore, 1980) and neoplastic cells (Wilson et al, 1980).

3.1.2 GENERAL PROPERTIES

Plasminogen activator catalyzes the conversion of the circulating zymogen plasminogen to another neutral protease, plasmin, a broadly active trypsin-like subunit which activates enzyme cascades likely to be important in inflammation. Plasminogen is capable of degrading fibrin to give rise to fibrin degradation products for which macrophages have receptors; activating complement components C1 and C3; generating kinins and cleaving activated Hageman factor to subunits that increase the formation of of kallikerein from prekallikrein (Goldstein, 1983, Nathan,

1980). Plasmin activates collagenase (Werb et al, 1977); collagenase and elastase can degrade components of vessel walls, perivascular tissue and articular surfaces.

The major murine plasminogen activator is a diisopropyl phosphofluoridate (DFP) - sensitive serine protease, MW 48:000, and is very close to the comparable enzymes released by virally transformed mouse fibroblasts. Macrophages release a second plasminogen activator, MW 28,000 that is also a serine enzyme. Studies identified the plasminogen activators as trypsin-like enzymes which are stable in culture at 37^{o}C for at least 48 hours (Unkeless, Gordon and Reich, 1974). Mechanisms of release of plasminogen activator, lysozomal hydrolases and lysozyme are independently regulated since the enzymes are secreted according to different kinetics and responses to modifications of macrophage activity are different (Baggiolini and Schnyder, 1982). Also, they appear to be confined to different subcellular compartments.

Plasminogen activator has recently been shown to exist in two active forms in inflammatory macrophages: a soluble form released - probably from secretory vesicles - into the extracellular medium and a cell-associated form which is membrane bound. Release of the membrane bound enzyme is likely to occur by a shedding mechanism rather than by exocytosis (Solomon et al, 1980; Lemaire, Drapier and Petit, 1983).

3.1.3 FUNCTION OF PLASMINOGEN ACTIVATOR

Plasminogen activator has a major role in the inflammatory response and, in conjunction with the other neutral proteases, could provide a mechanism for the central role of the macrophage in the pathogenesis of chronic inflammatory disease. The conversion of plasminogen to plasmin generates fibrinopeptides and active complement components capable of increasing vascular permeability and chemotactic for macrophages and PMN's. These pathways generate peptides that are pharmacologically active. Products of collagen degradation elicit macrophages so that it appears that plasminogen activator could promote further accumulation of macrophages, aid their migration from blood to tissues, permit contact with other cells and enhance macrophage activity in the site of inflammation (Vassali, 1976; Nathan, 1980; Baggiolini and Schnyder, 1982; Gordon, 1976).

Secretion of proteolytic enzymes will also degrade unwanted proteins, permit removal of fibrin debris in wounds by phagocytes and contribute to regulating the environment surrounding the macrophage in inflammation and tissue repair (Unanue, 1976).

Two separate stimuli appear to be required to trigger macrophage synthesis and secretion of PA (Gordon, Unkeless and Cohn, 1974). The first is a preparatory stage that can be achieved by inoculation of the animals with small doses of a lipopolysaccharide endotoxin. Cells from these animals release little PA, however phagocytosis of particles such as latex induces marked enzyme secretion (Cohn, 1975). It is possible that

the first stimulus in some way functions to rearrange molecules of the plasma membrane so that contact of a second triggering stimulus is able to occur (Ogmundsdottir and Weir, 1980).

Secretion of the enzyme declines after digestion of phagocytosed particles. However, persistance of undegraded particles is associated with prolonged fibrinolytic activity which could generate a chronic granulomatous response (Gordon, Nathan and Bloom, 1978).

Plasminogen activator is able to attack a number of extracellular substrates, so extensive control mechanisms are present to regulate production and to extend or restrict their range of activity (Gordon, 1980). Excessive activity is normally prevented by the naturally occurring inhibitors such as glucocorticoids and alpha 2 macroglobulin (Vassali, Hamilton and Reich, 1976; Nathan, Murray and Cohn, 1980). Secretion of enzyme in excess of these inhibitors could give rise to tissue injury associated with chronic inflammatory disease.

In vitro secretion of plasminogen activator by inflammatory macrophages can be inhibited by steroids, mitotic inhibitors and agents related to cyclic-AMP (C-AMP) and its metabolism. Secretion of the enzyme can be induced and stimulated in vitro by lectins such as Concanavalin A (Con A), the tumor promotor and potent inflammatory agent phorbol myristate acetate (PMA) and by secretion products of activated lymphoid cells (Vassali, Hamilton and Reich 1976; Vassali, Hamilton and Reich, 1977; Klimetzek

and Sorg,1977 and Vassali and Reich, 1977). By modulating stimulatory and inhibitory influences enzyme production can be modulated continuously over a 200-fold range (Vassali, 1980 : 1305).

3.1.4 THE CHOICE OF PLASMINOGEN ACTIVATOR RELEASE AS A PARAMETER IN THIS STUDY

Plasminogen activator secretion was selected as a parameter in this study because release of the enzyme is a well established marker of macrophage activation (Baggiolini and Schnyder, 1982; Gordon, 1980 : 1275; Cohn, 1978).

Numerous innovative studies have shown that macrophages release plasminogen activator after exposure to various inflammatory, immunologic and endocytic stimuli (Gordon, 1980 : 1275), whereas resident peritoneal macrophages show minimal PA activity (Unkeless, Gordon and Cohn, 1974; Gordon and Cohn, 1978). Unkeless, Gordon and Cohn (1974) were the first workers to show that macrophages elicited by an inflammatory stimulus, such as intraperitoneal injection of thioglycolate medium, synthesize and secrete large amounts of plasminogen activator. A large number of studies followed this observation, and showed that plasminogen activator is released by macrophages after exposure to asbestos (Hamilton et al, 1976); or by microbial infection such as Trypanasoma cruzi (Nogueira, Gordon and Cohn, 1977a) or BCG (Gordon and Cohn, 1978) followed by a secondary challenge with homologous antigen. PA release can be induced in resident

peritoneal macrophages by *in vitro* exposure to Con A and PMA (Vassali, Hamilton and Reich, 1977), by lymphocyte conditioned medium (Gordon and Cohn, 1978; Newman et al, 1978; Nogueira, Gordon and Cohn, 1977b) and by methylene blue (Schnyder and Baggiolini, 1980). Control of synthesis and secretion is achieved by the presence of serum components (Drapier et al, 1979; Chapman, Vavrin and Hibbs, 1979). Release is often stimulated by phagocytosis (Gordon, Unkeless and Cohn, 1974) or by *in vitro* exposure to Con A or PMA and inhibited by colchicine, vinblastine, cholera toxin and glucocorticoids (Vassali, Hamilton and Reich, 1976).

Mackaness's studies (1964, 1967) showed that during infection with facultative intracellular bacteria, cell-mediated immunity is induced and antimicrobial resistance and delayed type hypersensitivty arise simultaneously, and coincide with morphological and functional changes in the macrophages of the diseased host. Mackaness (1970) concluded that macrophage activation occurs as a result of the infection and leads to the destruction and elimination of facultatively intracellular bacteria. Chronic microbial infections such as tuberculosis are established because of the organisms ability to resist destruction within macrophages. Organisms such as *M.tuberculosis* and the nocardiae must be able to neutralize host defence mechanisms in order to survive and establish infection.

Infection of compromised individuals by opportunistic microbes such as *Nocardia* may perhaps be explained by the theory that

macrophage function is altered or becomes deficient as a result of the disease or immunosuppressive treatment of the host (Beaman, 1976:394). Plasminogen activator release, an index of macrophage function, was selected as a method of determining whether macrophage function is enhanced or deficient with respect to this index in Nocardia asteroides and/or Nocardia brasiliensis infections.

In most studies, the amount of PA released by the stimulated macrophage appears to correlate directly with the degree of stimulation (Chapman, Vavrin and Hibbs, 1982). Enzyme induction is a graded response (Gordon, 1980 : 1282) and there is a correlation between the production of PA and the inflammatory response in the peritoneal cavity (Hamilton, 1980). Measurement of macrophage plasminogen activator release would therefore give an indication of the inflammatory response to Nocardia asteroides and Nocardia brasiliensis infections. Nocardia infections are typically chronic and progressive (Beaman, 1976:387) and plasminogen activator has been shown to play a major role in the inflammatory response. Plasminogen activator secretion may be perpetuated by undergradable microbial constituents (intracellular parasites are primarily non-digestible particles [Katoh, 1981]) and lead to chronic activation of the coagulation system and production of kinins which are strongly associated with the development of inflammatory lesions and tissue injury (Hamilton, 1980).

Gordon and Cohn (1978 - see 3.3.1.9 for more detail) showed that

stimulation of macrophage plasminogen activator by PPD antigens depended on specifically sensitized T-lymphocytes, since it was abolished by pre-treatment with anti-thy 1.2 antiserum and complement. The experimental system developed in this study provided a simple, sensitive and versatile assay to study the role of lymphocytes and specific antigen in macrophage activation by BCG (Gordon and Cohn, 1978). Ortiz-Ortiz et al (1979) showed that an extract of N.brasiliensis is a B-cell mitogen; PPD is also a B-cell mitogen, so we wanted to investigate whether extracts such as culture filtrate antigens of N.asteroides and N.brasiliensis would modify macrophage responses in vitro. In addition, Folb et al (unpublished observations) found that exposure of lymphocytes in vitro to culture filtrate antigens of N.asteroides and N.brasiliensis significantly inhibited DNA synthesis in these cells (see 3.3.1.5.2). Folb, Jaffe and Altmann (1976) had previously suggested that the pathological effects of N.brasiliensis may be related to inactivation of normal T-lymphocyte function by the organism. They subsequently showed that the T-lymphocyte is an essential component in normal host resistance to infection by both N.asteroides and N.brasiliensis (Folb, Timme and Horowitz, 1977). Since then several investigations have established that T-cells are critical in host resistance to systemic and chronic nocardial infections (Beaman and Sugar, 1983). In order to determine whether macrophage responses in vitro might be modulated by secondary challenge with specific antigen, a study of macrophage plasminogen activator release was undertaken. The investigation attempted to elucidate the role of T-cells in the activation of

macrophages in experimental Nocardia infections.

The parameter of plasminogen activator release was used to determine whether macrophage responses in the two infections are different, to assess macrophage responses early in infection and to investigate whether macrophage function is modulated over the 21 day post-inoculation period, as discussed under 1.9.

3.2 MATERIALS AND METHODS FOR ASSAY OF PLASMINOGEN ACTIVATOR

Levels of plasminogen activator in cultures of unstimulated macrophages are barely detectable, however relatively high levels of enzyme are secreted by inflammatory macrophages. Even so, sensitive assays are needed to detect the enzyme since it is released in trace amounts. The methods used were those described by Gordon (1978) and Gordon, Werb and Cohn (1976).

3.2.1 PRINCIPLE

Plasminogen activators catalyze the conversion of plasminogen to plasmin, which in turn breaks down fibrin into fibrin degradation products. The enzymatic cleavage reactions involved in fibrinolysis are shown in Fig 4.

The plasmin activity generated by activated macrophages is regulated by the inhibitors alpha 2 macroglobulin, alpha 1 antitrypsin antithrombin III and C_1 inactivator, and amplified by Hageman factor, kininogen and complement (Gordon, 1978).

A simple procedure to assay activity of plasmin from zones of lysis in fibrin plates was first developed by Astrup (1975). Unkeless et al (1973) modified this procedure by using ^{125}I - labelled fibrin tissue culture dishes and in this way developed an assay with increased sensitivity and adaptability. A thin film of ^{125}I -labelled fibrinogen is baked on the bottom of tissue culture dishes and the fibrinogen is converted to fibrin by the action of thrombin. Plasminogen activator released by intact activated macrophages or in cell free fractions generates plasmin and this is measured from solubization of the ^{125}I fibrin.

3.2.2. REAGENTS

3.2.2.1 PREPARATION OF PURIFIED PLASMINOGEN (PLG) AND PLASMINOGEN-FREE SERUM

Purified plasminogen was prepared according to the method of Deutsch and Mertz (1970) from human serum and dog serum by affinity chromatography on lysine-sepharose.

3.2.2.1.1 Preparation of lysine sepharose

Briefly, Sepharose 4B (Pharmacia Fine Chemicals, AB Uppsala, Sweden) was suspended in 100 ml distilled water, washed three times by settling and decantation to remove azide and then re-suspended in 100 ml distilled water. 5g of Cyanogen Bromide (CNBr) was dissolved in 40 ml of N-methyl-2-pyrrolidone and the gel was activated by adding the CNBr using an autoburette with the endpoint of the titrator set to pH 11. When the reaction was complete the gel was washed with distilled water and then with

coupling buffer (Ø.1M NaH CO_3, pH 9.5). The gel was sucked free of excess liquid then suspended in 4°C coupling buffer containing 2Øg of ligand (L-lysine monohydrochloride) and tumbled overnight at 4°C. The agarose was then washed with water and unreacted imidocarbonate groups on the gel were masked by tumbling with 1M ethanolamine (pH 9) overnight. The gel was then washed and packed loosely into a column.

3.2.2.1.2 Chromatography

Filtered serum was run through the affinity column at 4°C and the plasminogen-free serum collected, and aliquoted and stored at -2Ø°C. After washing the column thoroughly with Ø.3M phosphate buffer, pH 7.2 the adsorbed plasminogen was then eluted with a solution containing Ø.2M Epsilon amino caproic acid (EACA) mixed 1:1 with Ø.1M phosphate buffer pH 7.2. The EACA was removed by dialysis against phosphate buffered saline (PBS). The plasminogen was then treated with diisopropyl phosphofluridate (DFP, Sigma Chemical Company, St Louis, MO 63178, USA)) i.e. the solution was made 1Ø mM with respect to DFP and incubated at 4Ø°C for 2 hours to inactivate traces of contaminating plasmin. Unreacted DFP was extracted by extensive dialysis against Ø.1M Tris-HCl, pH 8.1. Each batch of plasminogen was assayed against urokinase to determine the optimal concentration required for assay. The plasminogen was aliquoted and stored at -2Ø°C.

3.2.2.2 UROKINASE (UK)

Purified UK was obtained from Leo Pharmaceutical Products (Ballerup, Denmark), in the form of a lyophilised powder. The UK

was dissolved in 0.1M Tris-HCl, pH 8.1, containing 8 mg/ml bovine serum albumin (BSA) to give a stock solution of 2000 Ploug units/ml. The solution was acid and DFP treated (as described under 3.2.2.3) and stored in 50 microlitre aliquots in microfuge tubes at -80°C. Each aliquot was thawed and used once only. Each new batch of UK was standardized in the PA assay against the previous batch.

3.2.2.3 BOVINE SERUM ALBUMIN (BSA)

BSA (Miles Scientific, Division of Miles Laboratories Inc., Naperville, Il 60566, USA) was dissolved in water to give a solution of 20 mg/ml. Acid-labile protease inhibitors were removed by acid treatment : the solution was adjusted to pH 3.0 with 3N HCl, incubated at room temperature for 2 hours and neutralized with 2N Sodium hydroxide (NaOH).

Protease activity was removed by DFP treatment. DFP was added to the solution to give a final DFP concentration of 10 mM and then incubated at 40°C for 1 hour, then the procedure was repeated. Unreacted DFP was removed by extensive dialysis against distilled water. BSA was diluted to 8 mg/ml, aliquoted and stored at -20°C.

3.2.2.4 ^{125}I-FIBRIN PLATES

3.2.2.4.1 Purification of fibrinogen

Bovine fibrinogen (Fraction 1, Sigma Chemical Co.) was purified by a modification of Laki's procedure (1951) and by ethanol precipitation in the presence of lysine to reduce plasminogen

contamination (Mosesson, 1962).

Briefly, 2g fibrinogen was dissolved in 100 ml 0.1M KPO_4 buffer pH 6.4, the volume was made up to 200 ml with distilled water and the solution left overnight at 4°C. The solution was centifuged, the pellet discarded, and 100ml saturated ammonium sulphate added to precipitate the fibrinogen. The mixture was stirred slowly for an hour at room temperature, centifuged, the pellet dissolved in 50ml 0.6M NaCl and the pH adjusted to 7.4 with dilute NH_4OH. The solution was dialysed against 0.6M NaCl at room temperature. One part fibrinogen solution was diluted with 5 parts 0.12M lysine HCl in 0.005M $NaHPO_4$ pH 7.0, cooled at 0°C, any precipitate that formed was discarded, then the fibrinogen was precipitated by adding 8ml of 95% ethanol and dissolved in 30 ml 0.6M NaCl. The ethanol precipitation was repeated, the final precipitate collected by centrifugation, dissolved in 0.6M NaCl and dialysed against PBS. The fibrinogen solution was prefiltered, then sterilized by filtration, the OD_{280} was read and the fibrinogen concentration adjusted to 10 mg/ml. Fibrinogen was aliquoted and stored at -20°C.

3.2.2.4.2 Preparation of ^{125}I Fibrinogen plates

^{125}I fibrinogen coated Linbro multiwell plates (Flow Laboratories Ltd, Irvine, Scotland, Cat no. FB-16-24-TC) were kindly prepared and supplied by the Department of Clinical Science and Immunology, Medical School, University of Cape Town. ^{125}I fibrinogen coated linbro plates were prepared with purified fibrinogen as described by Strickland and Beers (1976).

Labelling of the fibrinogen with ^{125}I was performed by the method of Helmkamp et al, 1960, with the modification that each well contained 100 000 - 130 000 cpm releasable by trypsin (Wilson and Dowdle, 1978). 200 microlitres of a 5 mM NaCl solution containing 30 micrograms of ^{125}I fibrinogen was added to each well. The plates were then incubated at 45°C for 48 hours to dry the solution and ensure firm attachment of the fibrinogen to the well surface.

3.2.2.4.3 Activation of plates before use

^{125}I fibrinogen was converted to ^{125}I fibrin immediately before use by incubating each linbro well with 1ml Dulbecco's medium + 5% FCS for 2 hours at 37°C. Wells were then washed twice with HBSS (Direct Assay) or twice with PBS and once with Tris-HCl, pH 8.1 (Indirect Assay) to remove any unbound ^{125}I fibrinogen, which usually amounted to 20-30 000 cpm. Residual liquid in the wells was removed by suction.

3.2.2.5 TRYPSIN (DIFCO)

2.5g of Trypsin (Difco Laboratories, Detroit, Mi. 48232, USA) was dissolved in 500 ml 0.001 N HCl, prefiltered and 1ml 0,5% phenol red added. The solution was millipored through a 0.45 micron filter and was diluted to 0.25% in sterile TD, double strength, pH 7.4 (16.0g NaCl, 0.76g KCl; 0.25g $Na_2 HPO_4 . 2H_20$; 6.0g Tris per litre = double strength TD) with 0.02% EDTA. The solution was aliquoted and stored at -20°C.

3.2.3 CULTURE OF MACROPHAGES

Peritoneal cells were plated on Falcon petri dishes to assay for plasminogen activator secreted into the medium, or on ^{125}I-fibrin coated linbro wells for the assay of fibrinolysis by intact cells.

The procedures for culture of macrophages described in Chapter 2 were followed for the determination of plasminogen activator released into conditioned medium. 2 x 10^6 macrophages were cultured in sterile petri dishes in 15% ATFBS + M, P+S in DB; with or without various concentrations of Nocardia culture filtrate antigen. Macrophages from mice inoculated with N.asteroides organisms were exposed in vitro to N.asteroides antigen and cells from animals inoculated with N.brasiliensis were exposed to N.brasiliensis antigen, at concentrations of Ø.Ø2% and Ø.ØØ2% for 48 hours.

Standard culture procedures were modified in the assay for macrophage fibrinolysis. 5 x 10^5 macrophages were distributed into ^{125}I fibrin-coated linbro wells in DB supplemented with 2% FCS and P+S, with or without appropriate antigen, in a total volume of 1.5 ml. In cultures of macrophages elicited with thioglycolate medium it was necessary to suppress fibrinolysis by incubating cells in 6Ø micrograms /ml soybean trypsin inhibitor (STI; Sigma Chemical Co.) in DB supplemented with 5% HIFCS and P+S. The plates were washed free of lymphocytes and the cells fed fresh medium after 24 or 48 hours incubation. It is important to ensure that fibrinolysis is not due to contamination

with PMN, which are highly active. PMN die within 6-12 hours in culture so the cells were cultured for at least 12 hours before fibrinolysis assay (Gordon, 1978:33:10).

3.2.4 COLLECTION OF CONDITIONED MEDIUM (CM)

CM was routinely prepared after 48 hours cultivation on regular tissue culture dishes. The monolayers were washed twice with HBSS (37°C) to remove lymphocytes and inhibitors. Macrophages were incubated in 1 ml serum-free DB supplemented with 0.2% lactalbumin hydrolysate (LAH, Sigma Chemical Co.) for 24 hours. 50 microlitres of 8 mg/ml BSA was added to each tube prior to collection of CM. The CM was collected, centrifuged at a maximum relative centrifugal force of 2633 and the supernatant stored at -20°C until assay (Gordon, 1978; 33.6). PA is sticky and is easily lost on surfaces especially when handling dilute solutions in serum-free medium, so BSA was added to minimize enzyme losses. PA activity is stable over several cycles of freezing and thawing (Gordon, 1978: 33.6, 33.7).

3.2.5 CELL LYSATES

Macrophage monolayers were washed twice with PBS (37°C), lysed in 1000 microlitres of 0.5% Triton X-100. Tissue culture dishes were frozen and thawed once and scraped with a plastic policeman. Cell lysates were stored in Beckman microfuge tubes (Beckman Instruments Inc., Palo Alto, California 94304) at -20°C until Fluram assay (described in 2.3).

3.2.6 ASSAY OF FIBRINOLYSIS

In this procedure a solid-phase substrate was provided by radioactive fibrin coated as a thin layer on the bottom inside surface of linbro multiwell plates. Cells or CM to be assayed were added to the wells and PA activity was measured as plasminogen-dependent solubilisation of radioactivity as a function of time. The assay was conducted at 37°C in a humid 5% CO_2 environment .

The direct assay of fibrinolysis is done on intact cells plated on ^{125}I fibrin coated wells. This method measures both secreted and cell membrane-associated plasminogen activator (Jones, Goldfarb and Holden, 1983). The indirect assay is performed for cell-free fractions which have been secreted into the tissue culture medium (Hamilton, 1983).

3.2.6.1 DIRECT ASSAY : INTACT CELLS

3.2.6.1.1 Methods for calibration and routine experiments

3.2.6.1.1.1 FCS as a source of plasminogen

Murine macrophages were elicited by intraperitoneal injection of Brewers thioglycolate medium (See Chapter 2.7) and harvested as described previously. Resident macrophages were harvested from mice inoculated with normal saline only. After 20 hours in culture, a 100 microlitre sample was withdrawn from each linbro well and the amount of ^{125}I fibrin solubilised during incubation determined using a Beckman Gamma Counter (Model 4000, Beckman Instruments Inc., Scientific Instruments Division, Irvine Ca, 92713). Macrophage monolayers were washed twice with TD (37°C) and measurement of fibrinolysis was begun by placing cells in DB

+ 5% ATHIFCS in the presence or absence of Concanavalin A (Con A, British Drug Houses, Poole, England). Fibrinolysis was estimated by withdrawing 100 microlitre aliquots at various times and assaying them for solubilised radioactivity.

3.2.6.1.1.2 Dog Serum (DS) as a source of plasminogen

Monolayers were washed twice with HBSS (37°) after the desired incubation period and after monitoring degree of fibrinolysis during incubation. Fibrinolysis was initiated by incubation in 1 ml of DB + 5% ATDS + P+S. 100 microlitre aliquots were with drawn at intervals (1-6 hours) and assayed for released radioactivity.

3.2.6.1.1.3 Assay using purified plasminogen (PLG)

This method is identical to that described under 3.2.6.1.1.2 except that purified PLG (10 micrograms/ml) was substituted for ATDS (Gordon, 1978: 33.7).

3.2.6.1.2 General considerations for the assay of fibrinolyis by intact cells

Fibrinolysis was prevented during the incubation period by the use of inhibitors - Soya Bean Trypsin Inhibitor (STI, Sigma Chemical Company) was used for thioglycolate-elicited macrophages and 2% FCS for unstimulated and Nocardia-activated cells. Media were monitored for release of radioactivity before each change of medium.

Cell morphology was monitored at various intervals during the

assay by phase-contrast microscopy. Macrophages attach and spread well on fibrin (Gordon, 1978; 33.7). Cultures were pre-incubated to ensure loss of PMN and were washed twice before measurement of fibrinolysis to remove inhibitors and most of the lymphocytes.

Appropriate controls were used in all experiments : duplicate wells without cells were always included and the plasminogen dependence of fibrinolysis was tested by the use of plasminogen-depleted sera. In the experiments using purified plasminogen, addition of the zymogen was omitted in appropriate wells. Plasminogen dependence of fibrinolysis was confirmed by reconstitution with plasminogen.

Results were expressed as the percentage of total radioactivity releasable by trypsin. The percentage trypsin value was obtained by determining the cumulative radioactivity released into each well over the three separate time points. The percentage radio-activity released at each separate time point relative to the total radioactivity released by trypsin could be calculated from this. Background levels of fibrinolysis (no cells plated) were usually low.

In a representative number of experiments, the washes prior to initiation of fibrinolysis were collected from individual wells, concentrated by centrifugation and an aliquot counted for number of macrophages. This was done to determine whether macrophage losses at this point were large and whether losses of stimulated

and unstimulated macrophages were similar. In a similar number of randomly selected experiments the viability of plated macrophages was checked by addition of dilute Trypan blue (Chapter 2.8.3) to cultures after wells have been washed and excess fluid sucked away.

Several concentrations of macrophages were plated on the ^{125}I-fibrin and fibrinolysis assayed to ensure that fibrinolytic activity was proportional to cell number. Also, the optimal concentration of AT serum or purified plasminogen was determined. Duplicate cultures were assayed in each experiment at least, and as many as 8 replicate cultures were assayed where possible (depending on the available number of macrophages).

Fibrinolysis was monitored by measuring the solubilized radioactivity in 100 microlitre aliquots at three separate timepoints.

3.2.6.2 INDIRECT ASSAY : SECRETED ENZYME

The method employed for the determination of secreted PA is that described by Wilson and Dowdle (1978). Macrophages were preincubated in ATFCS as this results in higher levels of proteolytic activity in serum-free medium (Gordon, 1978; 33.8), CM was frozen at -20^{o}C until assay (Gordon, 1978; 1,33.6; Hamilton, 1983).

CM was thawed and maintained on ice immediately prior to assay. In each well, the final reaction volume of 300 microlitres

consisited of 10 microlitres of 8 mg/ml BSA (80 micrograms) 40 microlitres of 0.1 mg/ml PLG (4 micrograms),200 microlitres 0.1M Tris-HCl, pH 8.1 and 50 microlitres of CM added finally to initiate the reaction.

Appropriate controls were included in all assays. The plasminogen-independent proteolysis was detected by incubating the sample in the absence of PLG (Tris-HCl substituted), and background lysis due to plasmin contamination of the PLG solution measured in duplicate wells containing PLG without sample. The total radioactivity present in each well was determined by incubating 300 microlitres Trypsin solution in duplicate wells. Standard wells included in each assay contained UK in 14 doubling decrements, usually commencing with 0.05 Ploug units per well. UK was diluted in the same medium used to prepare CM.

Fifty microlitre aliquots of reaction mixture were removed at various time intervals - usually four separate time points were taken - and the solubilised radioactivity measured in a gamma counter.

For each time point, the UK standard curve was plotted and sample values falling on the linear range of the standard curve were determined. Sample values were expressed as percentage of total trypsin activity (see 3.2.6.1.1.3) in terms of UK units per assay well. The average number of UK units was determined from the number of UK units calculated at the four separate time points. (Only sample values falling on the linear range of the

standard curve were used to calculate the average).

3.2.6.3 FLUORIMETRIC DETERMINATION OF TOTAL CELL PROTEIN

Total protein present in the petri dish after collection of CM was determined as described under 2.3. PA activity measured in the indirect assay was related the total cell protein value and results were expressed as milliunits of urokinase/mg of cell protein (mU UK/mg cell protein).

3.2.6.4. MOLECULAR WEIGHT OF PLASMINOGEN ACTIVATOR

The methods used were those described by Granelli-Piperno and Reich (1978) and Wilson et al, 1980.

Plasminogen activator present in CM was separated by means of sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). The SDS was removed from the gel after electrophoresis by washing with non-ionic detergent (2,5% Triton X-100). The gel was then layered onto a second indicator gel consisting of fibrin, plasminogen and agar. After incubation at 37°C, zones of fibrin degradation, corresponding to the position of proteases in the SDS-gel overlay, can be seen as clear areas of lysis under dark background illumination.

3.2.6.4.1 Polyacrylamide gel electrophoresis

The following buffers were used :

Running gel buffer (RGB) : 0,4% SDS in 1,5M Tris HCl pH 8.8

Stacking gel buffer (SGB): 0,4% SDS in 0.5M Tris HCl pH 6.8

Sample buffer : 1% SDS in 40% sucrose solution

Resevoir buffer : Ø.1% SDS in Ø.Ø25M Tris-HCl, Ø.192M Glycine pH 8.3.

14Ømm x 125mm Slab gels contained 12% polyacrylamide in the wick, 11% polyacrylamide in the running gel and 4% polyacrylamide in the stacking gel. The final concentration of SDS in the gel was Ø.1%. Catalysts added were TEMED (Ø.Ø5% in running and stacking gels) and ammonium persulphate $NH_4S_2Ø_3$ (Ø.Ø5% in the running gel and Ø.1% in the stacking gel). The samples contained a final concentration of 1% SDS.

Electrophoresis was performed at a constant current of 24 mA per gel until the dye front reached the bottom of the gel. Following electrophoresis, the track containing the molecular weight markers was removed and stained. The remainder of the gel was washed with gentle shaking for $1^1/_2$ hours in 2.5% Triton X-1ØØ, rinsed three times (1Ø minutes each time) in distilled water, dipped briefly into Ø.1M Tris-HCl buffer pH 8.1, drained and layered on the fibrin-plasminogen-agar indicator gel.

3.2.6.4.2 Fibrin-Plasminogen-agar gel

The fibrin-plasminogen-agar gels were cast between two clean glass plates, held Ø.8 mm apart by thin wires. The glass and wires were clamped together and warmed in a 45°C incubator. The gel solution contained 1.25% agar, 5 micrograms/ml of purified human plasminogen, 2 mg/ml of purified bovine fibrinogen and Ø.Ø6 units/ml of thrombin (Sigma Chemical Co., Bovine thrombin dissolved in Ø.5 ml gelatine buffer pH 7.8 [Ø.Ø5M sodium diethyl

barbiturate, Ø.1M NaCl and Ø.25% w/v gelatine] to give 2Ø N.I.H units/ml. This was stored in 5Ø microlitre aliquots at -2Ø°C) in Tris-HCl pH 8.1.

The agar was added to distilled water, boiled for 15 minutes, then transferred to a 45°C water bath. The solution of fibrinogen, plasminogen and thrombin were kept at 37°C, mixed, the mixture added rapidly to the agar and immediately pipetted between the prewarmed glass plates. The gel was allowed to solidify at room temperature then kept at 4°C in a humid chamber until used.

The SDS gel was layered onto the indicator gel, incubated at 37°C in a humid atmosphere and removed at intervals for viewing and photography. Bands of PA activity could be seen as clear zones of lysis in the cloudy background provided by the unlysed fibrin. Failure of fibrinolysis to occur in control indicator gels (plasminogen-free) was used as a criterion to define the enzymes as plasminogen activators.

The indicator gel was preserved by staining in Ø.1% amido black in 7Ø% methanol, 1Ø% acetic acid for 3Ø min. The gel was destained in 7Ø% methanol + 1Ø% acetic acid.

3.3 RESULTS

3.3.1 CALIBRATION OF THE EXPERIMENTAL SYSTEM

3.3.1.1 EFFECT OF INTRAPERITONEAL INJECTION OF THIOGLYCOLATE AND IN VITRO EXPOSURE TO CONCANAVALIN A ON MACROPHAGE PLASMINOGEN ACTIVATOR SECRETION

Various experiments performed by Vassali, Hamilton and Reich (1977) were repeated to ensure that the assay for PA set up in this laboratory could reproduce the results others had achieved. The ability to repeat the work of others would indicate correct preparation of reagents and setting up of the system.

3.3.1.1.1 Fibrinolysis by cultured macrophages exposed in vitro to Con A

The fibrinolytic activity of unstimulated macrophages was markedly enhanced by exposure to Con A in vitro. Maximal stimulation was achieved at a concentration of Con A of 10^{-7}M, (Table 12). These results are in accord with Vassali, Hamilton and Reich, 1977.

3.3.1.1.2 Thioglycolate elicted macrophages : effect of Con A exposure in vitro

Thioglycolate-elicited macrophages showed a high basal level of fibrinolytic activity, which was increased only slightly by Con A, fibrinolysis being maximal at a concentration of 10^{-7}M Con A, (Table 24a and b). These findings are in agreement with those of Vassali, Hamilton and Reich, 1977.

3.3.1.2 OPTIMISATION OF NUMBER OF MACROPHAGES DISTRIBUTED INTO EACH LINBRO WELL

3.3.1.2.1 Thioglycolate-elicited macrophages

Different numbers of thioglycolate-elicited peritoneal macrophages were distributed in duplicate linbro wells and incubated for 20 hours, media were monitored for breakthrough and cells were washed and plated in 5% ATHIFBS. Fibrinolysis was monitored at intervals over 48 hours. The range of macrophage numbers distributed in linbro wells was between $1,25 \times 10^5$ and 2×10^6 macrophages. Results obtained are shown in Table 13. 1×10^6 macrophages appear to produce maximum fibrinolysis under these experimental conditions, however distribution of 1×10^6 macrophages per well means that substantial numbers of cells are required for each experiment which would necessitate impractical numbers of mice being sacrificed. Microscopic examination of the cultures revealed that in the 1×10^6 macrophage wells, cells were crowded together expecially in the centre and only cells at the periphery spread well. In order to optimise experimental conditions and to achieve high levels of fibrinolysis, 5×10^5 macrophages were selected as a suitable number of cells to be distributed in each well.

To use this assay quantitively, it is necessary to determine conditions such that substrate digestion is proportional to the number of cultivated macrophages present, or to the amount of PA, i.e. conditioned medium used in the assay (Gordon, 1978 : 33.10). In this experiment, fibrinolysis increases with increase in cell number, i.e. amount of fibrinolysis is dependent on cell number, until a maximum of 1×10^6 macrophages per well. With

further increase in cell number, the graph shows a decrease in amount of fibrinolysis. This is possibly due to overcrowding of the cells, and nutrients also may become exhausted. A further possible explanation for the drop in fibrinolysis is that toxic metabolic products are produced which may build up, and although cultures may appear healthy by morphological criteria, normal cell function may not occur.

3.3.1.2.2 Nocardia-activated macrophages

The experiments with thioglycolate-elicited macrophages were repeated using different numbers of Nocardia-activated macrophages distributed in duplicate linbro wells. Macrophages were incubated in 2% FCS prior to induction of fibrinolysis, as described previously.

3.3.1.2.2.1 Fibrinolysis in the presence of ATDS

Fibrinolysis was measured using 5% ATDS as a source of plasminogen (Gordon and Cohn, 1978). Fibrinolysis was monitored over 6 hours. Table 14 shows the relation of fibrinolysis to cell number under these experimental conditions. Results are similar to those obtained for thioglycolate-elicited macrophages, therefore 5×10^5 macrophages was selected as a suitable number for assay of fibrinolysis in the presence of ATDS.

3.3.1.2.2.2 Fibrinolysis in the presence of purified plasminogen

Macrophage fibrinolysis was determined in the presence of 10 micrograms/ml purified plasminogen, which increases the

sensitivity of the assay (Gordon, 1978; 33.7). Fibrinolysis was monitored over 6 hours.

Results of a representative experiment using N.asteroides -activated macrophages are shown in Table 15a. The range of macrophage numbers distributed in linbro wells was between 2.5 x 10^5 and 1.5 x 10^6 macrophages. Cells were examined at intervals during the culture and assay period. It was noted that macrophages were well-spaced in the 2.5 x 10^5 culture wells; numerous cells were plated down in the 5 x 10^5 wells, and normal spreading had occurred. In the 7.5 x 10^5 macrophage wells, cells were spread and were confluent. Macrophages in the 1 x 10^6 wells were rather rounded, i.e. there was insufficient surface area available for spreading. Macrophages in the 1.5 x 10^6 wells were packed solid and were unable to spread on the bottom of the dish. The results shown in Table 15a are similar to those presented in Table 13.

Fibrinolysis increases with increase in cell number to a maximum with 1 x 10^6 macrophages per well. Further increase in cell number leads to a levelling off of amount of fibrinolysis. Similar experiments with N.brasiliensis-activated macrophages produced similar results : a representative experiment is presented in Table 15b.

On the basis of these findings, 5 x 10^5 macrophages was selected as the optimum number of cells to be distributed into each linbro well.

3.3.1.3 OPTIMISATION OF VOLUME OF CONDITIONED MEDIUM (CM) ADDED TO EACH LINBRO WELL AND PERIOD OF CULTURE IN SERUM-FREE MEDIUM

Nocardia-activated macrophages were incubated in Ø.25% LAH for 1 or 4 days. CM was collected at the end of the culture period and aliquots of 2Ø microlitres, 5Ø microlitres, 1ØØ microlitres, 2ØØ microlitres and 25Ø microlitres were assayed for PA activity. (Gordon, 1978; 33.6). Results for N.asteroides-activated macrophages are presented in Tables 16a and b. From this data it appears that 24 hours of incubation is sufficient - substantial fibrinolysis occurs with samples of CM collected after 24 hours in culture. In the direct assay, it is important to consider possible inactivation of the enzyme in the culture medium. Schnyder and Baggiolini (1978a) found that PA was inactivated rapidly after release. Unkeless, Gordon and Reich (1974) showed that PA in CM was stable at 37°C for at least 48 hours. 24 hours was selected in this series of experiments so that losses of PA would be minimised. In addition, 5Ø microlitres of CM collected after 4 days in culture rapidly released almost 1ØØ% of available radioactivity. Excessive solubilisation of the fibrin is undesirable since the graph describing fibrinolysis (% radioactivity released vs time) starts flattening off at high percentages of radioactivity released. Gordon (1978; 33.8) states that radioactivity released should be proportional to enzyme concentration and time of incubation so long as solubilization does not exceed about 3Ø% of the total count releasable by trypsin. Use of the urokinase standards allows a far greater degree of fibrinolysis to be acceptable.

Any enzyme values which fall outside the linear range of the UK standard curve are discarded since extrapolation at high and low levels of fibrinolysis is very inaccurate. In these experiments an upper limit of 80% and a lower limit of 4% was selected, even if the sample percentage trypsin lay on the linear part of the UK standard curve. However, since enzyme levels secreted by unstimulated macrophages are generally very low, enzyme levels below 4% trypsin are accepted for control cultures. From Table 16a it appears that 50 microlitres of CM is a suitable volume for assay. Increase in volume of CM assayed does not give rise to a proportionally greater degree of fibrinolysis, indeed at very high volumes, e.g. 250 microlitres of CM the amount of fibrinolysis decreases compared to e.g. 100 microlitres CM. This is probably due to the presence of inhibitors of fibrinolysis in the tissue culture medium, which in high volumes of CM may achieve inhibitory concentrations.

Experiments done using N.brasiliensis-activated macrophages produced similar results - a representative experiment is shown in Table 16c. Aliquots of 30 microlitres, 50 microlitres and 100 microlitres CM were assayed for PA activity. Fifty microlitres was selected as being a suitable volume of CM for assay.

In these experiments, the amount of fibrinolysis increases with increase in volume of CM assayed with a maximum at a volume of 100 microlitres. Fibrinolysis was therefore directly related to volume of CM added.

3.3.1.4 CHOICE OF PLASMINOGEN SOURCE IN ASSAYS FOR FIBRINOLYSIS

3.3.1.4.1 Comparison of responses in dog serum and in fetal calf serum

Macrophage fibrinolysis has been determined using AT-fetal calf serum (Vassali, Hamilton and Reich, 1977: Unkeless, Gordon and Reich, 1974), or AT-dog serum (Gordon and Cohn, 1978; Noguiera, Gordon and Cohn, 1977). AT-dog serum is used in the fibrinolytic assay for less active cells since FCS retains more antiplasmin activity than DS after acid-treatment (Gordon, 1978:33.6,33.7). The greater inhibitory effect of HIATFCS compared to ATDS is shown in Table 17a, in which fibrinolysis by thioglycolate-elicited macrophages is compared. Nocardia-activated macrophages and unstimulated control macrophages were assayed for fibrinolysis in both 5% ATFCS and 5% ATDS. Results are shown in Table 17b. After 12 hours of assay, fibrinolysis by N.brasiliensis-activated macrophages was negligible in the presence of ATFCS and was maximal in the presence of ATDS. ATDS was therefore selected as suitable for the fibrinolysis assay. Plasminogen-dependence of fibrinolysis was confirmed by incubating macrophages in ATFCS-P and ATDS-P - sera were depleted of plasminogen by two cycles of chromatography on lysine-sepharose (Tables 17b, 19).

3.3.1.4.2 Assay of fibrinolysis in the presence of acid-treated dog serum : response of unstimulated macrophages

Unstimulated macrophages do not release plasminogen activator

(Unkeless, Gordon and Cohn, 1974; Gordon and Cohn, 1978). Nogueira, Gordon and Cohn, (1977); and Gordon and Cohn (1978) investigated macrophage fibrinolysis in the presence of 5% ATDS. Unstimulated macrophages released baseline levels of PA over a six hour assay period. Examination of the results in Table 17a and in Table 17b reveals that the response of control, unstimulated macrophages to exposure to dog serum is different in the two experiments. High levels of fibrinolytic activity occurred in the one experiment (Table 17a) whereas baseline levels occurred in the other (Table 17b). The fibrinolytic assay in the presence of 5% ATDS was repeated many times, and in most experiments fibrinolysis produced by control macrophages exceeded or was similar to that produced by Nocardia-activated macrophages; although according to the literature, control macrophage fibrinolytic activity may be expected to remain at baseline levels over a six-hour assay period (Gordon and Cohn, 1978). Occasionally, for no obvious reason, fibrinolytic activity of unstimulated macrophages in the presence of ATDS was negligible. These inconsistent results lead to an investigation into the factors responsible for the occurrence of the phenomenon.

3.3.1.4.2.1 Fibrinolysis in the presence of AT-dog serum from different sources

ATDS used in all previous experiments was compared to a different batch of Gibco dog serum, also acid treated, and to dog serum obtained by bleeding a dog housed in the Animal House, Medical School, University of Cape Town. Blood was collected, the serum

separated by centrifugation, sterilised by filtration and acid treated.

Macrophages harvested from control mice were not pooled - 5 x 10^5 macrophages from individual mice were exposed to the three different batches of ATDS and fibrinolysis measured. Results are shown in Table 18. Plasmin backgrounds, i.e. fibrinolysis in wells incubated with serum but without cells, were low. Significant fibrinolysis occurred with all three batches of ATDS, therefore a particular batch of dog serum was not responsible for the phenomenon.

3.3.1.4.2.2 Plasminogen-dependence of fibrinolysis by unstimulated macrophages in the presence of ATDS

Unstimulated macrophages were assayed for fibrinolysis in the presence of ATDS and in ATDS from which the plasminogen had been extracted by chromatography on lysine-sepharose. Results are shown in Table 19. Fibrinolysis is strictly dependent on the presence of plasminogen.

3.3.1.4.2.3 Fibrinolysis by macrophages from individual mice in the presence of AT-dog serum

Macrophages harvested from mice were not pooled - 5 x 10^5 macrophages from individual mice were placed in linbro wells and fibrinolysis monitored in the presence of 5% ATDS.

In the experiment shown in Table 20, 20 control mice were used and the fibrinolysis produced by the macrophages from the

individual mice is presented. Macrophages from individual mice were incubated in 5% HIATFCS to compare responses. No detectable fibrinolysis occurred in the presence of HIATFCS. However, significant fibrinolysis was measured in the presence of ATDS.

Fibrinolysis by control macrophages occurs with macrophages from individual mice as well as with pooled macrophages. Pooling of macrophages is therefore not responsible for the high levels of fibrinolysis associated with control macrophages. When peritoneal washes from outbred strains of mice such as ICR are pooled, it is conceivable that each population of lymphocytes may react against "foreign" determinants on the surface of the other population and lead to a mixed lymphocyte reaction (MLR). This could in turn lead to macrophage activation and could, it was thought, account for the fibrinolysis produced by pooled control macrophages, since lymphocytes and macrophages were co-cultured for 48 hours prior to fibrinolysis assay. Serum plasmin inhibitors in ATFCS could mask the reaction.

The experiment presented in Table 20 shows that fibrinolysis occurs without pooling the macrophages, therefore a MLR is not responsible for the phenomenon.

3.3.1.4.2.4 Effect of removal of lymphocytes on unstimulated macrophage fibrinolysis in AT-dog serum

To determine whether unstimulated macrophage fibrinolysis in the presence of ATDS required the presence of lymphocytes, experiments were done in which peritoneal cell cultures were

washed twice with HBSS after two hours incubation to remove most of the lymphocytes. Macrophages were refed 2% FCS/DB/P+S and reincubated for 46 hours. Fibrinolysis was assayed in the presence of ATDS. Results of a representative experiment are shown in Table 21. Lymphocytes do not appear to be required - fibrinolysis by unstimulated macrophages occurs to a similar extent irrespective of whether lymphocytes are present during the 48 hours in culture or not. Control macrophages therefore do not require the presence of lymphocytes to become stimulated by contact with dog serum.

3.3.1.4.2.5 Control macrophages fibrinolysis in the presence of purified plasminogen

The possibility that control unstimulated macrophages become "activated" in culture was investigated. Schnyder and Baggiolini (1978a) found that plasminogen activator was released in very low but measurable quantities by unstimulated macrophages after 4 or 5 days of incubation, and they suggested that non-elicited macrophages may become activated in culture, since during the first 3 days no enzyme was detectable.

Many workers have implicated serum in the macrophage activation process. Drapier et al (1979) showed that <u>in vitro</u> secretion of PA by thioglycolate-elicited macrophages does not occur in the absence of serum - secretion of soluble activator is blocked and cell-associated activator accumulates intracellularly (Lemaire, Drapier and Petit, 1983; Drapier, Lemaire and Petit, 1982) - however a short exposure to serum after 2-3 days of cultivation

in serum-free medium induced marked enzyme release. Serum therefore acts as a stimulus for PA release by thioglycolate-elicited macrophages. These workers (1982) state that the secretion of PA into the extracellular medium is modulated by various serum components.

Lejeune et al (1978) found that the presence of serum in macrophage cultures may be responsible for "non-specific" activation. They showed that normal heat-inactivated sera from various species can render unstimulated macrophages cytostatic, and can amplify the cytostatic properties of macrophages from immunised animals.

Cohn and co-workers (1965, 1966) established that serum is an essential substance for inducing pinocytosis and production of lysozomal enzymes by macrophages. Opitz et al (1977) demonstrated that fetal calf serum contains a factor which can support an in vitro primary immune response. Reikvam, Gammeltvedt and Hoiby (1975), found that peritoneal macrophages can be stimulated in vitro by new born calf serum and show an increase in content of lysozomes, lysozomal enzymes and phagocytosis.

Sherman, Lee and Stewart (1981) investigated the hypothesis that macrophages could be stimulated to release PA by the specific substrate, viz fibrin. Unstimulated macrophages were incubated in vitro with soluble fibrin/fibrinogen complexes for 24, 48 and 72 hours. These cells released significant

proteolytic activity in 24 hours, and fibrinolysis increased with increased time of incubation with soluble substrate.

In the experimental system described in this chapter, macrophages are in contact with fibrin which is gradually solubilising for 48 hours prior to the assay for fibrinolysis (solubilisation during the incubation period is monitored - see 3.2.6.1.2 and is minimal, however gradual solubilisation of the fibrin does occur - Gordon, 1978; 33.7). Release of PA by unstimulated macrophages under these conditions is therefore a distinct possibility.

To determine whether serum components or contact with fibrin or both were responsible for the marked secretion of PA by unstimulated macrophages in the presence of ATDS, fibrinolysis was measured using purified plasminogen instead of ATDS. Addition of purified plasminogen to intact cells has the advantage of rendering the assay considerably more sensitive (Gordon, 1978:33.7; Gordon, 1980:129).

Unstimulated and Nocardia-activated macrophages were incubated for 48 hours in 2% FCS/DB + P+S and assayed for fibrinolysis in the presence of 10 micrograms/ml purified human plasminogen (Gordon, 1978; 33.8) or in 5% ATDS. The plasminogen concentration was arbitrarily selected from the data presented by Gordon (1978:33.8).

Substantial fibrinolysis occurred with this concentration of

plasminogen; in Table 15a and b, plasminogen concentration did not appear to be limiting since fibrinolysis was proportional to cell number, therefore 1Ø micrograms/ml plasminogen was selected as suitable for the fibrinolysis assay.

Results of a representative expermiment are shown in Table 22. Plasminogen-dependence of fibrinolysis was confirmed. The unstimulated macrophages give rise to significant fibrinolytic activity - with plasminogen this was expected since Gordon (1978, 33.8) states that resident macrophages that have been cultivated for 24 hours will lyse a substantial amount of the fibrin in the presence of plasminogen. However, unstimulated cells lysed far less fibrin in the presence of plasminogen than in ATDS. Control macrophages generally released higher percentages of available radioactivity than Nocardia-activated macrophages in the presence of ATDS; in the presence of purified plasminogen this relationship is reversed. This reversal of the relationship was confirmed in numerous repetitive experiments in the direct assay for fibrinolysis, and in the indirect assay for secreted enzyme (Section 3.3.2). In the assay for secreted enzyme, barely detectable levels of fibrinolysis were produced by control macrophages. Macrophages from the same pool of cells used in the assay for fibrinolysis (Table 23a) were used in the assay for enzyme secretion (indirect assay, 3.2.6.2) and results are shown in Table 23b. Significant fibrinolysis occurred with the unstimulated macrophages cultured on fibrin but not with CM from unstimulated macrophages. Reasons for this are the much greater sensitivity of the assay for fibrinolysis, also significant

fibrinolytic activity occurs with cells cultivated for 24 hours on fibrin and assayed in purified plasminogen, probably due to contact with soluble fibrin.

From this data is appears that the high degree of fibrinolysis produced by unstimulated cells may be attributed to a large extent to dog serum components, whereas cultivation of cells on fibrin contributed to a much lesser extent.

Purified plasminogen was selected as the most suitable source of plasminogen in the assay for fibrinolysis by intact cells.

3.3.1.5 SELECTION OF APPROPRIATE MOUSE STRAIN

3.3.1.5.1 Results of thioglycolate and Concanavalin A Experiments

Experiments were done using macrophages from outbred (Ha) ICR/UCT mice and the inbred mouse strains BALB/c/UCT, CBA/Ca/UCT and C_3H/He/UCT.

The appropriate mouse strain for use in the assay for fibrinolysis was determined by measuring macrophage responses to intraperitoneal injection of thioglycolate medium and to incubation with Con A *in vitro*, as described previously.
Results of a representative experiment are shown in Tables 24a and b. Each value shown is the average of duplicate readings. Macrophages from CBA/Ca/UCT mice responded poorly to stimulation with thioglycolate medium and also to *in vitro* exposure to Con A. Macrophages from the other three mouse strains degraded the

fibrin to different extents, (Ha)ICR/UCT mouse macrophages causing less fibinolysis than C_3H/He/UCT macrophages and more fibrinolysis than BALB/c/UCT macrophages. The CBA/Ca/UCT strain was considered unresponsive and therefore unsuitable, and the strain producing the intermediate response i.e. (Ha) ICR/UCT was selected.

3.3.1.5.2 Results of histopathological and experimental studies of lymphocyte responses

Folb, Jaffe and Altmann (1976) investigated N.asteroides and N.brasiliensis infections in mice and used Swiss white mice in their studies. They established a model for N.asterioides and N.brasiliensis infections in Swiss white mice and found from serial histopathological studies that the lesions produced by the two organisms have quite different features (refer to section 1.7.3.1.1). An acute suppurative abscess characterised the lesions of N.asteroides, whereas N.brasiliensis caused a granuloma in which a striking feature was the presence of numerous "foamy" macrophages. The finding that the local pathogenic response to these two species of Nocardia is quite different despite relatively similar biochemical and antigenic characteristics, lead to the idea that cellular immunity may be depressed in N.brasiliensis infection. This possibility has been investigated in this study. Folb et al (personal communication) studied Swiss white mouse splenic T- and B-lymphocyte responses to culture filtrate antigens of N.asteroides and N.brasiliensis. Lymphocyte responses to antigen exposure were monitored by measurement of uptake of ^3H-thymidine. Effect of in vitro

homologous antigen exposure on lymphocyte DNA synthesis was measured in control mice and in mice previously inoculated intraperitoneally with live N.asteroides or N.brasiliensis organisms. These workers found that antigens of N.asteroides had no significant effect on uptake of ^{3}H-thymidine by T-lymphocytes harvested from control or N.asteroides-inoculated mice, however normal B-lymphocyte uptake of ^{3}H-thymidine was significantly depressed by 2% N.asteroides antigen. Depression of B-lymphocyte DNA synthesis was also shown with lymphocytes from mice previously inoculated with N.asteroides.

Normal T-lymphocyte responses of Swiss white mice were depressed by 2% N.brasiliensis antigens. Prior inoculation to Swiss white mice with N.brasiliensis did not modify this finding. Similarly, B-lymphocyte reponses were depressed by N.brasiliensis antigens in normal and N.brasiliensis-inoculated mice. The authors concluded that N.asteroides and N.brasiliensis culture filtrate antigens elicited different in vitro T- and B- lymphocyte responses in Swiss White mice as a result of antigenic differences between the two organisms. They postulated that depression of lymphocyte responses may be due to a T-cell-dependent suppressive influences, resulting in an overall hyporesponsiveness to high concentrations of antigens of N.brasiliensis. The (Ha)ICR mouse strain is derived from the Swiss White mouse, therefore it was appropriate to use that particular strain in this study which is a continuation of the studies of Folb et al.

3.3.1.6 NEGATIVE CONTROL STUDIES

3.3.1.6.1 Plasminogen-Dependence of Fibrinolysis

Plasminogen-dependence of fibrinolysis was routinely checked in both the assay for fibrinolysis and in the assay for secreted PA. Intact cells were assayed in the presence of serum depleted of plasminogen by two cycles of chromatography on lysine-sepharose or purified plasminogen was replaced with culture medium.

In the assay for secreted enzyme, plasminogen was replaced by Tris. In all experiments, fibrinolysis was strictly dependent on the presence of plasminogen and therefore due to macrophage PA.

3.3.1.6.2 Background fibrinolysis in the absence of cells

In all assays, duplicate linbro wells were assayed for fibrinolysis in the absence of cells. Background levels of fibrinolysis were generally low in assays with purified plasminogen, after 4 hours (median = 1.955% fibrinolysis, lower limit 1.15%, upper limit 2.474%, n = 22) and a median % Trypsin value of 6.948% with ATDS, after 4 hours (lower limit 4.499%, upper limit 9.718%, n = 22) of assay. Fibrinolysis was therefore strictly dependent on the presence of macrophages. Significant fibrinolysis in the absence of cells is indicative of plasminogen contaminated with plasmin. Plasmin is removed by treatment with DFP, as described under 3.2.2.1. Background levels of fibrinolysis were subtracted from all sample values, before calculation of % Trypsin values.

3.3.1.6.3 Evidence that there is minimal spontaneous loss of radioactivity during the incubation period

Media were monitored for "breakthrough" of fibrinolysis before each change of medium. An aliquot of medium was removed from each linbro well immediately prior to washing, and counted to determine released radioactivity. Percentages of fibrinolysis that occurred during the 48 hours in culture for a typical experiment are shown in Table 25. In general, less than 10% of available radioactivity was lysed during the pre-assay culture period.

3.3.1.7 CELL VIABILITY AND INDICATION OF CELL LOSSES DURING WASHES

Equal numbers of control and Nocardia-activated macrophages were distributed into linbro wells and incubated for 48 hours. Immediately prior to assay for fibrinolysis, cells were monitored for "breakthrough" of fibrinolysis and washed twice to remove lymphocytes and inhibitors.

Cell washes for each linbro well were collected, concentrated by centrifugation, resuspended in 500 microlitres of medium and macrophages were counted as described previously. These counts were done to find out whether cell losses were similar in the different experimental groups. Cell loss from the groups was minimal and there did not appear to be a group which consistently had greater loss of macrophages than another group. Results of six representative experiments are shown in Table 26.

Viability of macrophages was assessed after the final time point by washing cells, wells were sucked dry and dilute Trypan blue (Ø.1%) added. Cells were examined for uptake of dye.

In the fields of cells examined, occasionally one cell had stained blue. This indicates that cell viability was good at the end of the culture period.

3.3.1.8 RELATIVE CELL NUMBER AFTER INCUBATION

Various methods were employed to assess cell number after the culture period. Initially, Versene solution (Ø.Ø2% w/v EDTA in normal saline, Flow Laboratories, Irvine, Scotland # 28-2Ø3-49) was used to remove adherent cells. After the final timepoint, media was removed by suction. 1 ml of versene added to the well and the dish placed at $4^{o}C$ for 15 minutes. The well surface was scraped with a plastic policeman, the cells were checked microscopically and appeared no longer adherent. The medium was removed, pelleted and resuspended and an aliquot placed in a haemocytometer and examined microscopically. Although several wells were treated in this way, no cells were seen on the haemocytometer.

An attempt was then made to count the number of cells removed with 1 ml versene using a coulter counter. 5ØØ microlitres of cell suspension in versene was diluted to 1Ø millilitres in isoton (Coulter Electronics, Hialeah, Florida, USA) and Ø.5 ml of this counted in a coulter counter (model ZBI, Coulter Electronics). Results of a representative experiment are shown in

Table 27.

Cell numbers from control and Nocardia-activated macrophage wells were relatively similar, however, cell yields with versene treatment was low. In order to increase the number of cells recovered, lidocaine, 10.7 mM (Remedia (Pty) Ltd, Johannesburg, SA)/EDTA, 3mM (Merck Darmstadt, Germany) was used (van der Meer et al, 1978). Cells were treated with 1 ml of lidocaine/EDTA for 15 minutes at room temperature, observed microscopically and when they appeared to have detatched were scraped from the surface of the well as vigorous rinsing of the surface with jets of media from a pasteur pipette did not seem to dislodge cells. 500 microlitres of cell suspension were added to 9.5 ml of isoton and a 500 microlitre aliquot counted in a coulter counter. Although a counting plateau (calibration curve of the range of cell sizes in the sample - at high values this curve plateaus off) could be established for the control cells, it could not be obtained for the Nocardia-activated cells, therefore it appeared that lidocaine/EDTA was unsuitable as a cell-dislodging agent. Nathan and Root (1977) found that lidocaine was not suitable for the resuspension of adherent BCG-activated macrophages because these cells were far more tightly adherent than cells from untreated mice and an accurate count could not be obtained.

The next approach was to measure total cell protein at the end of the incubation period. Cells were washed twice with PBS and lysed with 1000 microlitres 0.5% Triton X-100 and frozen and

thawed (Biggar and Sturgess, 1977). The Lowry method (Lowry et al, 1951) was used. However, a yellow precipitate formed between the Triton X-100 and the Folin-Ciocalteau's Phenol reagent. Total cell protein was then measured using the fluorimetric method described in 2.3. This method was selected for routine use. The low cell counts recovered with versene and a plastic policeman are similar to those described by others. Van der Meer et al (1978) state that about 25% of cells are recovered using this method and about 60% using lidocaine/EDTA. Although cell yields were low, cell numbers were similar for control and Nocardia-activated macrophages, indicating that the ability of both groups of cells to survive in culture was similar, and that any differences in enzyme release was probably not related to differences in cell number.

Schnyder and Baggiolini (1978a) say that specific activities in the culture media may be related to actual cell numbers or to the amount of cellular protein. They prefer to express enzyme activities per 10^6 cells, since total cell protein may drop as a result of release of enzyme or may markedly increase as the cell becomes activated. In the literature both methods are widely used (Gordon and Cohn, 1978; Unkeless, Gordon and Cohn, 1974; Vassali, Hamilton and Reich, 1977; Nogueira, Gordon and Cohn, 1977a; Schnyder and Baggiolini, 1978a; Hamilton, 1980; Hamilton, 1983). Nathan, Karnovsky and David (1971) selected cell protein rather than cell number for comparing macrophage activities so that differences would not simply reflect possible differences in cell size.

Activated macrophages are generally increased in size, which is expressed in their increased protein content (Cohn, 1978). However, studies have shown protein content to remain the same (Stubbs et al, 1973) or to be decreased (Kondo and Kanai, 1977). From results presented in section 8.3.9 it appears that protein content of control cells is similar to that of Nocardia-activated cells. Calibration experiments indicate that Nocardia -activated macrophages release more PA than unstimulated macrophages. Nocardia-activated macrophages appear larger than unstimulated macrophages on microscopical examination (Ch 5 and 9). However this apparent difference is not statistically significant except for control and N.brasiliensis-activated macrophages in the elongated form (significant at the Ø.Ø3 level, Mann Whitney U Test. Data from SEM study, section 9.3). Median values and their 95% confidence limits for macrophage size are shown in Table 117. If Nocardia-activated cells did have increased protein content, specific activity of PA expressed as a conservative estimate of quantitative enzyme differences in the two types of macrophages. Because of the difficulties encountered in recovering the cells from the surface into suspension, the fluorimetric method for determination of total cell protein was selected, and secreted PA was expressed in terms of total protein.

3.3.1.9 ADDITION OF CULTURE FILTRATE ANTIGENS OF N.ASTEROIDES AND N.BRASILIENSIS IN VITRO

Gordon and Cohn (1978) measured fibrinolytic activity of

peritoneal macrophages obtained from mice 3 weeks after infection with viable BCG organisms and showed only a twofold increase in fibrinolytic activity. However, when the peritoneal cells from infected animals were incubated *in vitro* with different amounts of either of two soluble antigen preparations, purified protein derivative (PPD) antigen and *M.tuberculosis* culture filtrate for 1 or 2 days, a striking dose-related enhancement of macrophage fibrinolysis resulted. Experiments showed that two different preparations of antigen, PPD and *M.tuberculosis* culture filtrate could both be used for challenge *in vitro*.

In order to determine whether secondary challenge with specific antigen *in vitro* modulated *Nocardia*-activated macrophage release of PA, unstimulated macrophages and *Nocardia*-activated macrophages were incubated with various concentrations of homologous culture filtrate antigen (i.e. macrophages from mice previously inoculated with *N.asteroides* organisms were exposed to *N.asteroides* antigen *in vitro* and macrophages from mice previously inoculated with *N.brasiliensis* organisms were exposed to *N.brasiliensis* antigen *in vitro*). The method of Gordon and Cohn (1978) was followed.

3.3.1.9.1 *Nocardia asteroides* antigen : dose response and time response curves

A number of experiments were performed to determine the optimal dose of antigen and time of incubation with antigen. These experiments were done with macrophages from mice inoculated 2,

7, 13 and 21 days previously with N.asteroides. Representative experiments are shown in Tables 28 and 29. Macrophages from mice inoculated 7 days previously with N.asteroides were incubated with various concentrations of N.asteroides culture filtrate antigen (range Ø.2% to Ø.ØØØØ2%) for 24, 48 or 72 hours.

Time of incubation did not appear to influence macrophage responses to antigen. However, macrophages incubated for 48 hours released a greater percentage of the available radioactivity than macrophages incubated for 24 hours (Table 28). This observation is in agreement with that of Sherman, Lee and Stewart (1981). 72 hours of incubation was not selected because cells would be maintained on the fibrin for a total of 4 days. Gordon (1978, 33.7) states that it is not usually possible to culture cells on fibrin for more than 4-5 days because of gradual solubilisation of the fibrin; also contact of unstimulated cells with fibrin could lead to significant fibrinolytic activity, as occurred in the experiment shown in Table 29b. From these considerations, 48 hours was selected as the most suitable time of incubation with antigen.

There was no definite effect of the antigen noted in the calibration experiments. Ø.Ø2% and Ø.ØØ2% antigen concentrations were selected as being the most suitable dose to use in subsequent experiments since Ø.2% antigen is a concentration which would be unlikely to occur physiologically and Ø.ØØØ2% and Ø.ØØØØ2% were tnought to be very low concentrations.

3.3.1.9.2 Nocardia brasiliensis antigen : dose response and time response curves

The experiments described under 3.3.1.9.1 were repeated for N.brasiliensis. Macrophages were harvested from mice inoculated previously with N.brasiliensis organisms and were exposed in vitro to N.brasiliensis culture filtrate antigens. Representative experiments are shown in Table 30. Macrophages from mice inoculated 13 days previously with N.brasiliensis organisms were incubated with N.brasiliensis antigen (range 0.2% to 0.00002%) for 48 and 72 hours. N.brasiliensis-activated macrophages released a higher percentage of available radioactivity after 72 hours of incubation. However, 48 hours was thought to be more suitable for the reasons given under 3.3.1.9.1.

Again there was no consistent antigen effect, so 0.02% and 0.002% antigens were arbitrarily selected as the doses to be used in subsequent experiments.

3.3.1.10 GELS: MOLECULAR WEIGHT OF PLASMINOGEN ACTIVATOR ASSAYED

The distances travelled by the molecular weight protein markers were measured and a calibration curve of log molecular weight vs refractive index plotted (Fig 5); (refractive index, RF = distance protein has migrated divided by length of gel).

In this experiment the length of the marker gel and the PA gel

was 13,4 cm. Bands of fibrinolysis were measured at 3,6 cm and 6,6 cm. These values correspond to RF values of Ø,269 and Ø,493. From the calibration curve, log molecular weights of 4,684 and 4,445 were obtained. The bands correspond to molecular weights of 48,3Ø5 and 27,861. In the literature, the major murine plasminogen activator band corresponds to a molecular weight of 48,ØØØ and the minor band to 28,ØØØ (Unkeless, Gordon and Cohn,1974). The enzyme(s) under study are therefore confirmed to be murine plasminogen activators.

3.3.2 PLASMINOGEN ACTIVATOR RELEASE BY PERITONEAL MACROPHAGES HARVESTED FROM MICE PREVIOUSLY INOCULATED WITH N.ASTEROIDES OR N.BRASILIENSIS

Release of plasminogen activator by macrophages from mice inoculated 2, 7, 13 or 21 days previously with N.asteroides or N.brasiliensis organisms or normal saline only, was measured using the direct assay for fibrinolysis by intact cells and the indirect assay for secreted enzyme.

The Mann Whitney U Test was used to test for statistically significant differences between the different experimental groups, ie control, control + Ø.Ø2% antigen, Nocardia-activated macrophages, Nocardia-activated macrophages exposed in vitro to Ø.Ø2% and Ø.ØØ2% antigen (section 2.9). Each of the different groups were compared with at each other for statistically significant differences at each of the three time points (T1, T2 and T3). For simplicity control macrophages were numbered group 1, control macrophages exposed to Ø.Ø2% antigen group 2,

Nocardia-activated macrophages group 3, Nocardia-activated macrophages exposed in vitro to Ø.Ø2% antigen group 4 and Nocardia-activated macrophages exposed in vitro to Ø.ØØ2% antigen group 5. This coding is used in both the text and in the tables.

In the figures, C = control; C* = control macrophages exposed in vitro to Ø.Ø2% antigen; Na = N.asteroides-activated macrophages; Na* = N.asteroides-activated macrophages exposed in vitro to Ø.Ø2% antigen; Na** = N.asteroides-activated macrophages exposed in vitro to Ø.ØØ2% antigen. A similar system was used for N.brasiliensis. Figures show median enzyme values and their 95% confidence limits. In addition, statistically significant differences, determined using the Mann Whitney U Test, between control macrophages and Nocardia-activated macrophages are shown on the figures. For levels of significance between control cells exposed to antigen and Nocardia-activated macrophages, refer to the relevant tables.

3.3.2.1 TWO DAY N.ASTEROIDES INFECTION

3.3.2.1.1 Direct assay for fibrinolysis

Three replicate experiments were performed, and the data are shown in Table 31.

Median percentage fibrinolysis values and their 95% confidence limits are shown in Table 32 and in Fig 6.

There was no statistically significant difference between the amount of fibrinolysis produced by control macrophages and that

produced by N.asteroides-activated macrophages at any of the three different time points. There was no significant effect of antigen exposure in vitro on control macrophages or Nocardia-activated macrophages, except at T3, where there was significantly less fibrinolysis produced by N.asteroides-activated macrophages compared with N.asteroides-activated macrophages exposed in vitro to Ø.Ø2% antigen ($p < Ø.Ø1$) and Ø.ØØ2% antigen ($p < Ø.Ø1$, Table 35).

3.3.2.1.2 Indirect assay for secreted plasminogen activator

Three replicate experiments were performed and the data are shown in Table 33.

Median milliunits of urokinase and specific activities of enzymes are shown in Table 34 and Fig 7 (median mU UK/2x1Ø^6 macrophages shown only). The Mann Whitney U Test was used to test for statistical difference (Table 35).

There was a borderline statistically significant difference between the number of milliunits of urokinase (mU UK) produced by control and N.asteroides-activated macrophages ($p = Ø.Ø5$), however there was no significant effect of antigen exposure in vitro on control or N.asteroides-activated macrophage enzyme secretion. There was a significant difference ($p < Ø.Ø5$) between control macrophages and N.asteroides-activated macrophages exposed to Ø.Ø2% antigen.

There was a statistically significant difference between the

specific activities of control and N.asteroides-activated macrophages ($p = 0.05$); of control and N.asteroides-activated exposed to 0.02% antigen ($p < 0.05$) and of control and N.asteroides-activated macrophages exposed to 0.02% and 0.002% antigen ($p < 0.05$ in both cases. Table 35).

3.3.2.2 TWO DAY N.BRASILIENSIS INFECTION

3.3.2.2.1 Direct assay for fibrinolysis

Five replicate experiments were performed, the data are shown in Table 36.

Median percentage fibrinolysis values and their 95% confidence limits are shown in Table 37 and Fig 8. N.brasiliensis-activated macrophages produced greatly increased levels of fibrinolysis (400% control), as did antigen-exposed N.brasiliensis-activated macrophages (0.02% : 390% control; 0.002% : 399% control. Percentages calculated from median fibrinolysis values). This finding is in agreement with the literature. There was a statistically significant difference between fibrinolysis produced by control and N.brasiliensis -activated macrophages ($p < 0.1$) at each time point; between control and N.brasiliensis-activated macrophages exposed to antigen (see Table 40 for levels of significance); between control macrophages exposed to antigen and N.brasiliensis -activated cells; and between antigen-exposed controls and antigen-exposed activated macrophages (see Table 40). There was no significant effect of antigen on control macrophages or on N.brasiliensis-activated macrophages.

3.3.2.2.2 Indirect assay for secreted plasminogen activator

Five replicate experiments were performed and the results are shown in Table 38.

Median enzyme values expressed as milliunits of urokinase and specific activities are shown in Table 39 and Fig 9. These results confirm the results of the direct assay (3.3.2.2.1) in that significantly more enzyme is secreted by N.brasiliensis -activated macrophages than by control macrophages. Similarly, significantly more enzyme was secreted by antigen-exposed N.brasiliensis-activated macrophages than by control cells or control cells exposed to antigen. This difference was significant whether expressed in terms of milliunits of urokinase or as specific activity, and levels of significance were all $p < 0.01$ (Table 40). There was no significant effect of antigen on control macrophages or on N.brasiliensis-activated macrophages, a result which concurs with the results of the direct assay.

3.3.2.3 SEVEN DAY N.ASTEROIDES INFECTION

3.3.2.3.1 Direct assay for fibrinolysis

Six replicate experiments were performed and the results are shown in table 41.

Median percentage fibrinolysis values, reflecting plasminogen activator levels, and their 95% confidence limits are shown in Table 42 and Fig 10. Fibrinolysis by N.asteroides-activated macrophages was significantly greater than that produced by

control macrophages and significanlty greater than that produced by antigen-exposed control cells ($p < 0.001$) at each time point. Similarly, antigen-exposed N.asteroides-activated macrophages released significantly more radioactivity than controls or antigen-exposed controls ($P < 0.001$ in each case, see Table 45). There was no significant effect of antigen exposure in vitro on control or N.asteroides-activated macrophages.

3.3.2.3.2 Indirect assay for secreted plasminogen activator

Seven replicate experiments were performed and the data are shown in Table 43.

Fig 11 and Table 44 show the median values for secreted plasminogen activator, expressed in terms of milliunits of urokinase and specific activity. The results are in agreement with the results obtained using the direct assay and are similar to those obtained for 2 day N.brasiliensis-activated macrophages. Significantly more plasminogen activator was secreted by N.asteroides-activated macrophages than by control cells or antigen-exposed control cells ($p < 0.001$ in each case). Similarly, significantly more plasminogen activator was released by antigen-exposed N.asteroides-activated macrophages than by control macrophages or antigen-exposed control macrophages (for levels of significance see Table 45). There was no significant effect of antigen exposure on control or N.asteroides-activated macrophages. Results are similar whether expressed in terms of milliunits of urokinase or specific activity.

3.3.2.4 SEVEN DAY N.BRASILIENSIS INFECTION

3.3.2.4.1 Direct assay for fibrinolysis

Cell-associated and soluble plasminogen activator release were measured in macrophages from mice inoculated seven days previously with N.brasiliensis in five replicate experiments. The data are shown in Table 46 and median enzyme values and their 95% confidence limits in Table 47 and Fig 12. Results are similar to those obtained with two day N.brasiliensis-activated macrophages and seven day N.asteroides-activated macrophages. Fibrinolyis was significantly increased in cultures of N.brasiliensis-activated macrophages compared to fibrinolysis produced by control macrophages and antigen-exposed control macrophages ($p < 0.001$ in each case, Table 50). Antigen-exposed N.brasiliensis-activated macrophages released significantly more plasminogen activator than control cells or antigen-exposed control cells (see Table 50). Antigen-exposure in vitro did not modify macrophage plasminogen activator release.

3.3.2.4.2 Indirect assay for secreted plasminogen activator

Results from six replicate experiments are shown in Table 48, and median plasminogen activator values and their 95% confidence limits are presented in Table 49 and Fig 13. The results concur with those obtained using the direct assay and are similar whether expressed in terms of milliunits of urokinase or specific activity.

Significantly more plasminogen activator was secreted by

N.brasiliensis-activated macrophages than by control cells or antigen-exposed control cells ($p < 0.001$ in each case). Also, significantly more enzyme was secreted by antigen-exposed N.brasiliensis-activated macrophages than control macrophages or antigen-exposed control cells ($p < 0.001$ in each case, Table 50). As shown with previous experiments, in vitro exposure to antigen did not modify control or N.brasiliensis-activated macrophage release of plasminogen activator. Results are similar whether expressed in terms of milliunits of urokinase or specific activity.

3.3.2.5 THIRTEEN DAY N.ASTEROIDES INFECTION

3.3.2.5.1 Direct assay for fibrinolysis

Fibrinolysis by thirteen day N.asteroides-activated macrophages was measured in four replicate experiments. Data from these experiments are shown in Table 51 and median enzyme values and their 95% confidence limits are shown in Table 52 and Fig 14. Significantly more plasminogen activator was released by N.asteroides-activated macrophages than by control or antigen-exposed control macrophages (except no significant difference was found at T3 for antigen-exposed controls and N.asteroides -activated macrophages, Table 55). There was a significant difference in the amount of enzyme produced by antigen-exposed N.asteroides-activated cells and that produced by control cells or antigen-exposed control cells at T1 and T2 ($p < 0.05$) but not at T3, except for control and 0.002% antigen exposed N.asteroides-activated cells ($p < 0.01$). In vitro antigen exposure did not modify plasminogen activator production by

control or N.asteroides-activated macrophages. Lack of significant differences at T3 may be attributed to wide scatter of data, in addition, control cells released quite large amounts of radioactivity probably as a result of contact with the fibrin.

3.3.2.5.2 Indirect assay for secreted plasminogen activator

The data from four replicate experiments are shown in Table 53, and median plasminogen activator values and their 95% confidence limits are given in Table 54 and Fig 15. Results are similar to those obtained in the direct assay.

More plasminogen activator was secreted by N.asteroides-activated macrophages than by control or antigen-exposed control cells (see Table 55). Also, significantly more enzyme was released by antigen-exposed N.asteroides-activated macrophages than by control macrophages or antigen-exposed control macrophages. Antigen exposure had no significant effect on plasminogen activator release by control macrophages. Results are similar whether expressed in terms of milliunits of urokinase or specific activity except in the case of N.asteroides-activated macrophages exposed to Ø.Ø2% antigen ($p < Ø.Ø5$).

3.3.2.6 THIRTEEN DAY N.BRASILIENSIS INFECTION

3.3.2.6.1 Direct assay for fibrinolysis

Five replicate experiments were performed and the data are shown in Table 56.

Median percentage fibrinolysis values and their 95% confidence

limits are shown in Table 57 and Fig 16. Results are similar to those obtained with two and seven day N.brasiliensis and seven day N.asteroides infections (thirteen day N.asteroides infection: only some significant differences were found at T3, Table 55). Fibrinolysis was significantly increased in cultures of N.brasiliensis-activated macrophages compared with fibrinolysis caused by control macrophages and antigen-exposed control macrophages (see Table 60 for levels of significance). Antigen-exposed N.brasiliensis-activated macrophages released significantly more plasminogen activator than control macrophages (except for 0.02% antigen which showed no significant difference at T3, Table 60), or antigen treated control macrophages. Antigen exposure in vitro did not modify macrophage plasminogen activator release, except at T2 for 0.02% antigen-exposed N.brasiliensis -activated macrophages.

3.3.2.6.2 Indirect assay for secreted plasminogen activator

Results from three replicate experiments are shown in Table 58 and median plasminogen activator values are shown in Table 59 and Fig 17.

Significantly more plasminogen activator was secreted by N.brasiliensis-activated macrophages than control macrophages or antigen-exposed control macrophages ($p < 0.01$, Table 60) when results were expressed in terms of urokinase units. When results were expressed in terms of specific activity, a borderline significant difference of $p = 0.05$ was achieved for control and N.brasiliensis-activated macrophages. A significant difference

was found between antigen-exposed control macrophages and N.brasiliensis-activated macrophages. Significantly more plasminogen activator was secreted by Ø.Ø2% antigen-exposed N.brasiliensis-activated macrophages than by control cells or antigen-exposed control cells ($p < Ø.Ø5$ in each case) but differences in levels of secreted plasminogen activator were not significant for Ø.ØØ2% antigen-exposed N.brasiliensis-activated macrophages. Antigen exposure had no significant effect on control macrophages or on N.brasiliensis-activated macrophages except when plasminogen activator levels for N.brasiliensis -activated cells were compared with levels for Ø.ØØ2% antigen-exposed N.brasiliensis-activated macrophages. The difference was significant ($p < Ø.Ø5$) when results were expressed in terms of urokinase but not when expressed as specific activity. Apart from this case, results are similar whether expressed in terms of milliunits of urokinase or as specific activity.

3.3.2.7 TWENTY-ONE DAY N.ASTEROIDES INFECTION

3.3.2.7.1 Direct assay for fibrinolysis

The data from three replicate experiments are presented in Table 61. Median percentage fibrinolysis and their 95% confidence limits are shown in Table 62 and Fig 18. No statistically significant differences in enzyme levels were found between any of the different groups at T3. Some significant differences were found at T1 and T2. Control and N.asteroides-activated macrophage enzyme levels were significantly different at T1 ($p < Ø.Ø5$); control and antigen-exposed N.asteroides-activated cells produced significantly different levels of enzyme at T1 and T2

(see Table 65 for statistics). Enzyme levels of antigen-exposed control cells were significantly different from those of N.asteroides-activated cells at T2 ($p < 0.05$). Antigen-exposed N.asteroides-activated macrophages produced significantly increased levels of fibrinolysis compared to control cells, except at T1 for antigen-exposed controls compared with 0.02% antigen-exposed N.asteroides, where no significant differences were shown. Antigen exposure in vitro did not modify control or N.asteroides-activated macrophage levels of fibrinolysis.

3.3.2.7.2 Indirect assay for secreted plasminogen activator

Table 63 shows the results from three replicate experiments and the median enzyme values and their 95% confidence limits are shown in Table 64 and Fig 19. Significantly more plasminogen activator was secreted by N.asteroides-activated macrophages than by control macrophages (Table 65) and by antigen-exposed N.asteroides-activated macrophages compared to control macrophages. These differences were statistically significant whether results were expressed in terms of urokinase units or specific activity. There was no significant difference in the amount of enzyme released by antigen-exposed N.asteroides -activated cells compared with the same cells not exposed to antigen. There was no significant effect of antigen exposure of control macrophages on enzyme release when results were expressed in terms of specific activity, but results were significantly different when expressed as milliunits of urokinase ($p < 0.05$). Antigen-exposed control cells were not significantly different from N.asteroides-activated cells or antigen-exposed

N.asteroides-activated macrophages in terms of plasminogen activator release except when results for antigen-exposed control and N.asteroides-activated cells were expressed in terms of specific activity ($p < 0.05$).

3.3.2.8 TWENTY ONE DAY N.BRASILIENSIS INFECTION

3.3.2.8.1 Direct assay for fibrinolysis

Cell-associated and soluble plasminogen activator levels were measured in five replicate experiments, data are shown in Table 66. Table 67 and Fig 20 shows median enzyme levels and their 95% confidence limits. At T1, no statistically significant differences were shown between enzyme levels in any of the different groups (Table 70). At T2 and T3 there was a significant difference between enzyme levels of control cells and N.brasiliensis-activated macrophages ($p < 0.05$) and between antigen-exposed control macrophages and N.brasiliensis-activated macrophages ($p < 0.05$). At T3, a significant difference ($p < 0.05$) was found between enzyme levels of N.brasiliensis-activated cells and the same cells exposed in vitro to 0.002% antigen.

3.3.2.8.2 Indirect assay for secreted plasminogen activator

Plasminogen activator secretion by 21 day N.brasiliensis -activated macrophages was measured in seven replicate experiments (Table 68). Median enzyme levels and their 95% confidence limits are shown in Table 69 and Fig 21. Results are similar to those of two day, seven day, and thirteen day N.brasiliensis-activated macrophages, but do not concur with the results of the twenty-one day N.brasiliensis direct assay. There

was no significant effect of antigen on control or N.brasiliensis -activated macrophages. Significantly more plasminogen activator was secreted by N.brasiliensis-activated macrophages than control ($p < 0.001$) or antigen-exposed control cells ($p < 0.001$, Table 70). Antigen-exposed N.brasiliensis-activated macrophages secreted more enzyme than control or antigen-exposed control cells. Results were similar whether expressed in terms of urokinase units or specific activity.

3.3.3 DIFFERENCES IN MACROPHAGE FUNCTION IN N.ASTEROIDES AND N.BRASILIENSIS INFECTIONS AND MODULATION OF PLASMINOGEN ACTIVATOR SECRETION OVER THE 21 DAY INFECTION PERIOD

The idea that plasminogen activator secretion may be different in N.asteroides and N.brasiliensis infection and modulated over the 21 day infection period was investigated by comparing enzyme values (expressed as percentage controls, Table 72). By inspection of this data, it appears that plasminogen activator secretion is maximal at 7 days post-inoculation in N.asteroides infection and at 2 days post-inoculation in N.brasiliensis infection. The pattern of secretion of plasminogen activator in N.asteroides infection appears to be different to that in N.brasiliensis infection and to be modulated over the 21 day period of infection in that enzyme release expressed as percentage control decreases over the 21 day infection period from a maximum at 2 days to a minimum at 21 days post-inoculation in N.brasiliensis infection; whereas percentage control enzyme levels are similar at 2 and 21 days, maximal at 7 days and

decreasing at 13 days, with a further decrease at 21 days, in N.asteroides infection. These trends are shown in Fig 22 (T2 only).

3.4 DISCUSSION

The experiments reported in this chapter indicate that macrophages from mice inoculated 2-21 days previously with N.asteroides or N.brasiliensis are activated with respect to the parameter of plasminogen activator release. However, the degree of macrophage response to the two infections appears to vary over the 21 day infection period. Therefore, macrophage function in the pathogenic Nocardia infections studied is not inhibited, it is enhanced with respect to plasminogen activator release over the 21 day period post-inoculation studied. Enhanced levels of plasminogen activator release occurred at 2, 7, 13 and 21 days post-inoculation for both N.asteroides and N.brasiliensis infections.

Macrophages from mice inoculated two days previously with N.asteroides do not release significantly more plasminogen activator than control macrophages when the direct assay method is used, however, borderline significant differences were shown between levels of enzyme released by the two different cell groups with the indirect assay method. In inflammatory macrophages, plasminogen activator occurs in two active forms (section 3.1.2). Cellular plasminogen activator is firmly membrane-bound and the formation of plasmin will be strictly dependent on the presence of the cells (Lemaire, Drapier and

Petit, 1983). The direct assay measures cell-bound and soluble plasminogen activator released by intact cells and the indirect assay measures enzyme secreted into the culture medium.

Macrophage activation generally results in a much greater increase in the amount of released than cell-associated plasminogen activator (Unkeless, Gordon and Reich, 1974; Solomon et al, 1980). Fibrinolytic proteolysis is most efficiently measured by direct attachment of cells to ^{125}I fibrin plates since both secreted and cell-membrane associated plasminogen activator is measured (Jones, Goldfarb and Holden, 1983). Under the assay conditions, unstimulated macrophages that have been cultivated on fibrin for 24 hours will release significant amounts of radioactivity (Gordon, 1978;33:7&8). Inspection of the data (Table 31) reveals that considerable fibrinolytic activity occurred with control macrophages, especially in experiment 3. Examination of records of post-mortem details and morphological data during culture did not reveal any unusual characteristics either for the mice or their macrophages, therefore control macrophage fibrinolysis was probably due to macrophage exposure to soluble fibrin (3.3.1.4.2.5, Sherman, Lee and Stewart, 1981), and the use of purified plasminogen. Although the direct assay is more sensitive than the indirect assay - in the indirect assay secreted plasminogen activator can only be measured under serum-free conditions (Jones, Goldfarb and Holden, 1983) - a serious drawback is the lack of standardization which makes it difficult to compare results with different batches of reagents (Gordon 1978; 33 : 10). This difficulty is overcome in the indirect assay by the use of urokinase reference

standards. It is likely that all these factors contributed to the different results obtained with the direct and indirect assay for 2 day N.asteroides-activated macrophages.

Macrophages harvested from mice 2 days post-inoculation with N.brasiliensis are activated with respect to the parameter of plasminogen activator release, compared to unstimulated macrophages whether the enzyme is measured by the direct or indirect method. Significant differences were shown at each time point (direct assay T1 -T3, $p < 0.01$) and results and statistics ($p < 0.01$) were similar whether indirect assay results were expressed in terms of urokinase units or specific activity.

Seven days post-inoculation with N.asteroides, harvested macrophages were activated by the criterion of plasminogen activator release compared to control macrophages whether the enzyme was measured directly or indirectly (direct assay, $p < 0.001$, indirect assay $p < 0.001$). Similar results were obtained for macrophages from mice inoculated seven days previously with N.brasiliensis (direct assay $p < 0.001$; indirect assay $p < 0.001$). Differences were significant at each time point in the direct assay and in the indirect assay and results and statistics were similar ($p < 0.001$ in each case) whether data was expressed as urokinase units or as specific activity.

Macrophages harvested from mice inoculated 13 days previously with N.asteroides release significantly increased amounts of plasminogen activator compared to control macrophages ($p < 0.05$,

direct assay; $p < 0.01$, indirect assay) and are therefore activated by this criterion. Similarly 13 day N.brasiliensis -activated macrophages are activated by the criterion of increased plasminogen activator release (direct assay, $p < 0.01$; indirect assay $p < 0.05$).

Macrophages harvested from mice inoculated 21 days previously with N.asteroides are activated with respect to the criterion of plasminogen activator release compared to control macrophages when enzyme levels are measured using the indirect assay ($p < 0.01$), but significant differences in plasminogen activator release between control and N.asteroides-activated cells were only found at T1 ($p < 0.05$) using the direct assay. Control macrophage percentages of fibrinolysis were fairly high in this series of experiments, reasons for this have been discussed earlier (discussion of results of 2 day N.asteroides-activated macrophages). In the indirect assay, results were similar and statistically significant whether expressed as urokinase units or specific activity.

In the series of experiments using macrophages harvested from mice inoculated 21 days previously with N.brasiliensis, macrophages were activated by the criterion of plasminogen activator release compared to control macrophages when the enzyme was measured by the indirect assay ($p < 0.001$) and by the direct method ($p < 0.05$) although results were not significantly different at T1. Inspection of this data (Table 66) reveals that 21 day N.brasiliensis-activated macrophages released rather low

levels of enzyme compared to macrophages from mice inoculated at the different time intervals prior to sacrifice.

To investigate whether plasminogen activator secretion is modulated over the 21 day infection period and whether degree of secretion is different in N.asteroides and N.brasiliensis infections, percentage control fibrinolysis values were calculated (direct assay). Enzyme secretion appeared to be maximal at 7 days post-inoculation in N.asteroides infection and at 2 days post-inoculation in N.brasiliensis infection. Secretion, in terms of percentage controls, appeared to be successively reduced over the 21 day period of infection with N.brasiliensis. In contrast, values for percentage secretion were low 2 days post-inoculation with N.asteroides, maximal at 7 days and thereafter successively reducing at 13 and 21 days to levels similar to those obtained at 2 days post-inoculation. Comparison of percentage control enzyme values for 2 day N.asteroides-activated macrophages with percentage control enzyme values obtained at 7, 13 and 21 days reveals that enzyme levels appear to be similar at 2 and 21 days and levels at 2 days are less than those obtained at 7 and 13 days. This apparent trend must be seen in context within the limitations of this assay (direct), since the stimulatory effects of the fibrin and purified plasminogen on control macrophages may be responsible for the low percentage control values obtained at 2 and 21 days post-inoculation, and this may be responsible for the apparent trend.

Percentage control values were calculated for the direct assay but not for the indirect assay since baseline levels of enzyme secretion are expected in the indirect assay for control cells, (Unkeless, Gordon and Reich, 1974) and this, in general, was found in the series of experiments employing the indirect assay. In fact, enzyme release from control cells frequently could not be detected. It was therefore felt that calculation of percentage controls for this assay was invalid since minor fluctuations of the baseline would result in dramatic changes in the percentage control value, with consequent modifications of the apparent trend of enzyme release.

There is a correlation between the production of plasminogen activator by the stimulated macrophage and the inflammatory response in the peritoneal activity (Hamilton, 1981; section 3.1.4). From percentage control enzyme values it appears that the inflammatory response to N.asteroides is greatest at 7 days, whereas inflammation is greatest at 2 days post-inoculation with N.brasiliensis. Thus, within the limitations of the assay, it appears that degree of macrophage response to N.asteroides and N.brasiliensis infection is different and is modulated over the 21 day infection period. This indicates that the inflammatory response in the peritoneal cavity is modulated over the period under study.

Macrophage activation by immunologically specific mechanisms occurs in the course of infection produced by intracellular organisms such as M.tuberculosis (Gordon, Newman and Bloom,

1978); and it has been shown in this study that infection with the facultative intracellular parasite Nocardia results in enhanced secretion of macrophage plasminogen activator. It has been stated that chronic inflammatory disease in the lung is prominently associated with stimuli that regulate the induction of macrophage proteinases in culture (Gordon, 1977), and that the ability to induce a sustained release of enzyme depended on the persistence of the phagocytized particle (Gordon, Newman and Bloom, 1978) - the decrease in fibrinolytic activity after particle digestion should limit and localize the generation of plasmin by macrophages (Gordon, 1978). Persistance of intracellular particles (such as intracellular parasites) is associated with continued generation of plasmin, collagenase and elastase and could serve to perpetuate a chronic granulomatous response (Gordon, 1977) such as occurs in Nocardia infection, eg in mycetoma.

Macrophages harvested from control mice or from N.asteroides or N.brasiliensis-inoculated mice were exposed to appropriate homologous culture filtrate antigens of Nocardia for 48 hours prior to the assay for fibrinolysis or incubation in serum-free medium.

Release of plasminogen activator by control macrophages was not significantly modified by in vitro exposure to Ø.Ø2% culture filtrate antigen except in one series of experiments - plasminogen activator release by 21 day N.asteroides-activated macrophages measured by the indirect assay. When data were

expressed in terms of urokinase units results were significant but not when expressed as specific activity. Inspection of the data (Table 63) reveals that antigen-exposed macrophages released large amounts of plasminogen activator in one experiment. The cell morphology data did not indicate any unusual characteristics for these macrophages in culture. Since neither the direct assay nor the indirect assay showed significant differences when data were expressed as specific activity, these results must be artifactual, statistical significance being achieved because of the low number of readings.

Nocardia-activated macrophages were exposed to 0.02% and 0.002% of appropriate homologous antigen and plasminogen activator release measured. In general, macrophage release of plasminogen activator was not significantly modified by exposure to antigen. However, significant effects of antigen exposure were found in a few instances. Using the direct assay for measurement of plasminogen activator, significantly different levels of fibrinolysis were found for 2 day N.asteroides-activated macrophages compared to the same cells exposed to 0.02% and 0.002% antigen($p < 0.01$) at T3 only. A reduction in percentage fibrinolysis occurred in both cases (median fibrinolysis percentages, from Table 32: N.asteroides-activated : 78.2%, N.asteroides-activated + 0.02%, antigen = 63.56%; N.asteroides -activated + 0.002% antigen = 62.67%), i.e. antigen exposure in vitro appears to inhibit plasminogen activator release by 2 day N.asteroides-activated macrophages. 21 day N.brasiliensis -activated macrophages released significantly less plasminogen

activator ($p < 0.05$) when exposed to 0.002% antigen *in vitro*.

Thirteen day *N.asteroides*-activated macrophages released significantly less plasminogen activator when exposed to 0.002% antigen *in vitro* when the enzyme was measured by the indirect assay and expressed in terms of specific activity. Results were not significant when expressed as milliunits of urokinase.

The apparent but irregular effect of antigen on plasminogen activator release led to the idea that antigen exposure *in vitro* did influence macrophage plasminogen activator secretion but that the effect was being masked by the pooling of data to test for statistically significant differences between groups. It was thought that this masking effect could be obviated by performing experiments with large numbers of replicate samples and testing for significant effects of antigen within a single experiment. Experiments were performed using 8 replicate samples in each group (i.e. control, *Nocardia*-activated, etc) in the direct assay. Statistically significant effects of antigen exposure were shown, however, these significant effects were not reproduced when the experiment was repeated, and consequently it was decided that antigen effects were erratic and no conclusions could be drawn from the results. It could be argued that the macrophages were already operating at peak capacity with respect to plasminogen activator secretion and could not therefore respond to a further stimulus. This was thought to be unlikely since comparison of plasminogen activator release by macrophages from mice inoculated 2 days previously with *N.asteroides* with

that of macrophages from mice injected 4 days previously with thioglycolate medium showed that thioglycolate-elicited macrophages lysed vastly greater amounts of the fibrin than N.asteroides-activated macrophages over the same time period (Table 17a).

Culture filtrate antigens of N.asteroides and N.brasiliensis were shown to be active and antigenic using a modification of the PCA skin test and by lymphocyte transformation tests (data not shown). One pathway for activating macrophages depends on the generation of specifically sensitized T-lymphocytes (Gordon and Cohn, 1978). It was hoped that the experimental model described by these workers would elucidate the role of T-cells in macrophage activation in experimental Nocardia infections. Experiments with unseparated lymphocytes, culture filtrate antigen and control and Nocardia-activated macrophages produced inconclusive results, so the attempt to determine the role of T-cells in macrophage activation was abandoned. It is possible that the culture filtrate antigens used in this study were inappropriate, and consequently no definite results were obtained. The role of T-cells in the activation of macrophages in experimental Nocardia infections remains to be determined.

In the direct assay, the range of pooled data within the groups is large (see 95% confidence limits). In a biological system, inter-experimental variation may be expected to be large, also the direct assay is not standardized, so that it is difficult to compare results with different batches of reagents (Gordon, 1978;

33 : 1Ø). Despite this variation, statistically significant difference were shown between data groups.

Several assays are available for measurement of plasminogen activator. However, all present limitations (Drapier, Vavrin and Hibbs, 1979). The ^{125}I-fibrin plate method (Unkeless et al, 1973) is the most sensitive (Drapier, Vavrin and Hibbs, 1979). There are several disadvantages of the ^{125}I-fibrin plate assay, the main drawback being the lack of standardization. Results obtained from discontinuous measurements may be obscured by plasmin-inactivation or by the presence of inhibitors (Drapier, Vavrin and Hibbs, 1979). Several difficulties are caused by the hydrolysis of fibrin - fibrinogen adsorbs fibrinolytic and thrombolytic factors so in the conversion of fibrinogen to fibrin using thrombin, the fibrin will contain adsorbed factors and the effect of this on protease systems is unknown. Fibrin-adsorbed plasminogen may decrease the sensitivity of the assay, and the insolubility of fibrin retards accomplishment of steady-state hydrolysis. These factors result in a logarithmic rather than linear assay (Kessner and Troll, 1976).

However, the ^{125}I-fibrin plate assay is rapid, sensitive and versatile; it can be used to detect plasminogen activator, plasminogen or plasmin in minute quantities. The assay can be made more or less sensitive by use of acid-treated serum or purified plasminogen so it can be tailored to individual experimental conditions. The main drawback of lack of standardization can be overcome by the use of urokinase reference

standards, and percentage fibrinolysis can be expressed in arbitrary units. It appears that in most studies of macrophage plasminogen activator release, workers have used the ^{125}I-fibrin plate method (Unkeless, Gordon and Reich, 1974; Gordon and Cohn, 1978; Vassali, Hamilton and Reich, 1977; Sherman, Lee and Stewart, 1981; Hamilton, 1980 and 1983). The vast amount of literature published on the subject gives a great deal of information on the use of and problems with the assay. Cell culture, in conjunction with the ^{125}I-fibrin plate assay, provides a useful closed system to study secretion in vitro, since it is feasible to study the effects of addition of antigens or mitogens such as Con A in vitro and it is possible to determine the intra- and extracellular distribution of plasminogen activator during continued cultivation (Gordon, 1976). The ^{125}I-fibrin assay was considered to be the most suitable method for determination of plasminogen activator in this experimental system.

Fig 4 : Enzymatic cleavage reactions involved in fibrinolysis

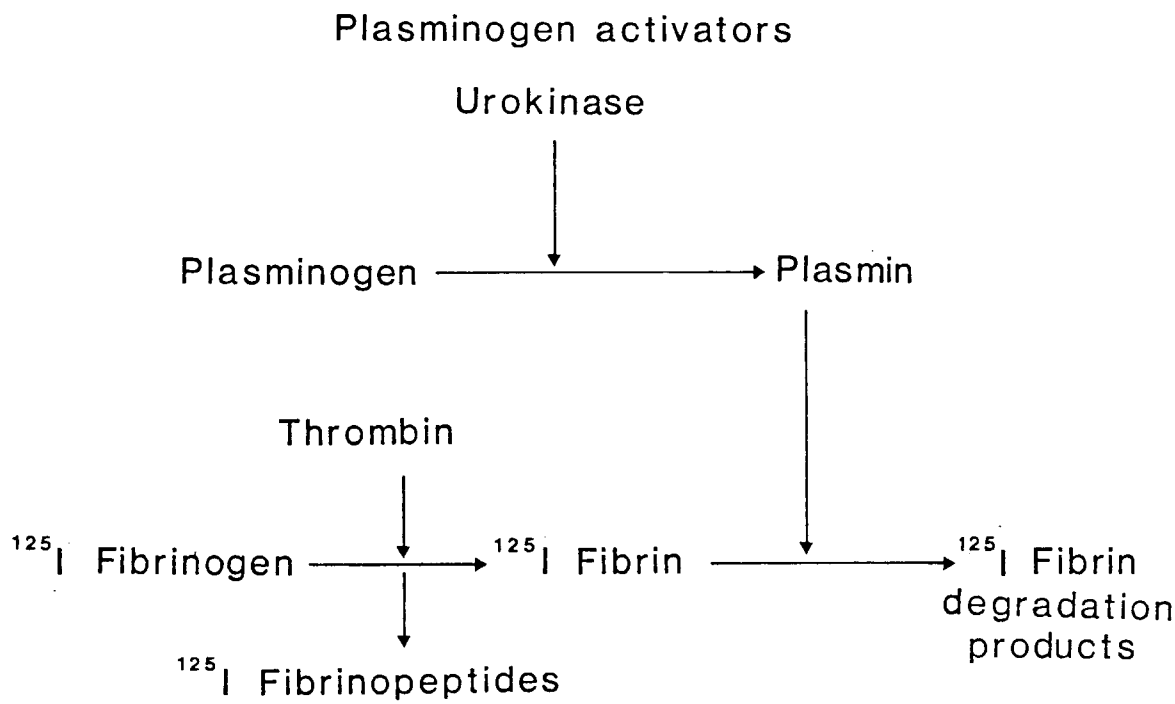


Fig 5 : Molecular weight of plasminogen activators : calibration curve of log molecular weight vs refractive index

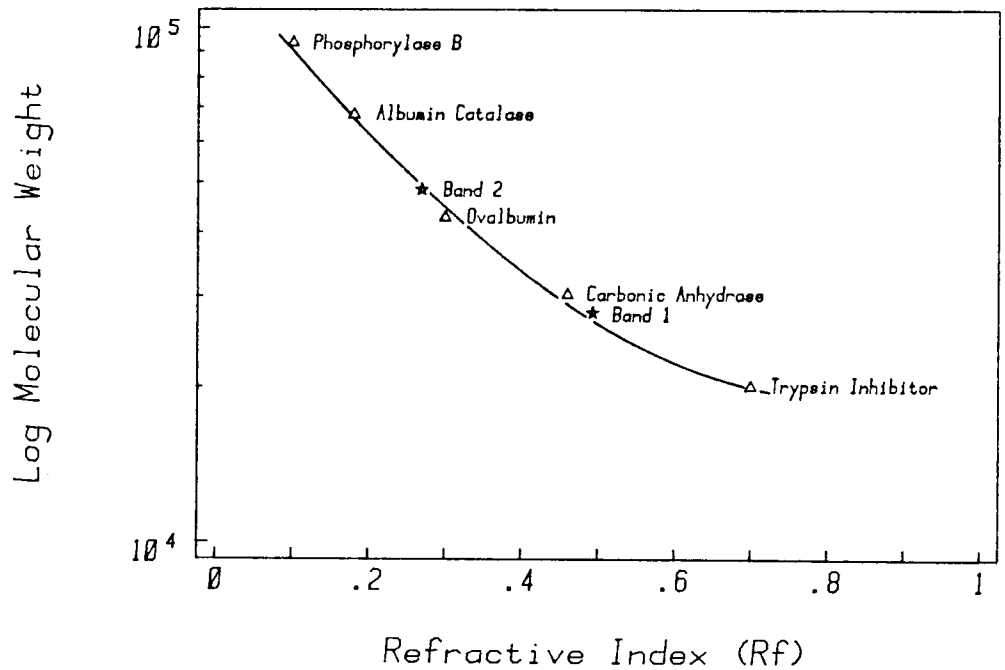


Fig 6 : Fibrinolysis produced by macrophages from mice inoculated 2 days previously with N.asteroides or with saline only : Direct assay

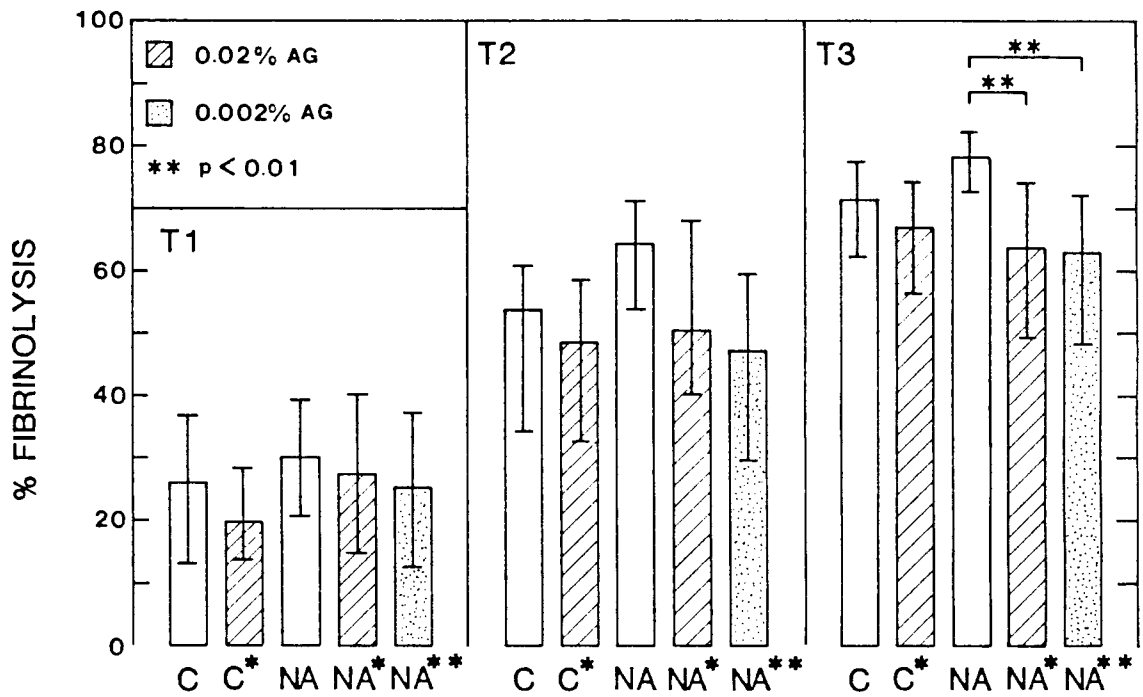


Fig 7 : Plasminogen activator secreted by macrophages from mice inoculated 2 days previously with N.asteroides or saline only : Indirect assay

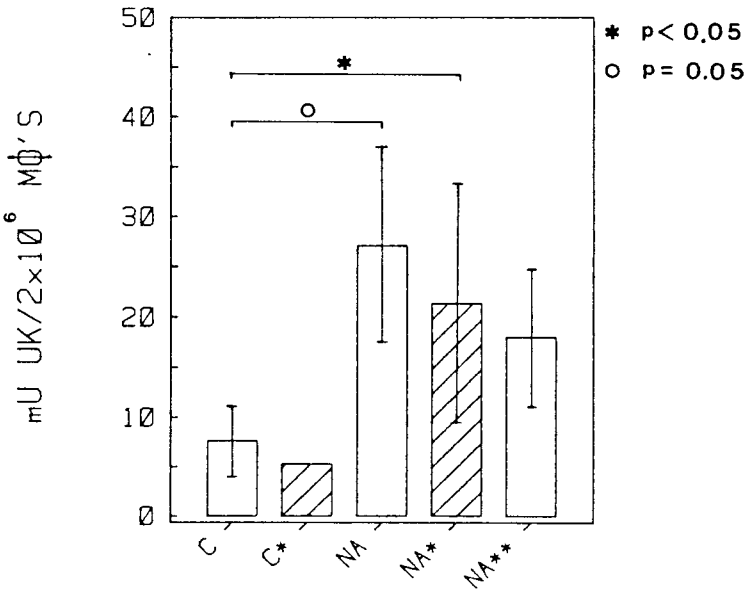


Fig 8: Fibrinolysis produced by macrophages from mice inoculated 2 days previously with N.brasiliensis or with saline only : Direct assay

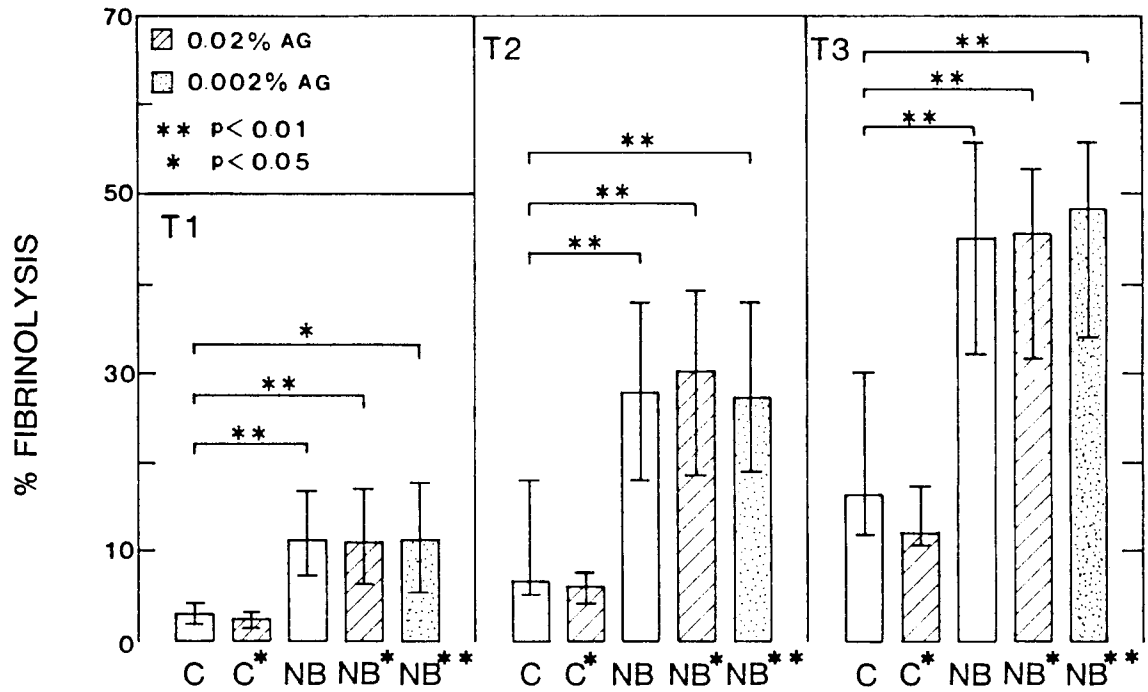


Fig 9 : Plasminogen activator secreted by macrophages from mice inoculated 2 days previously with N.brasiliensis or saline only : Indirect assay

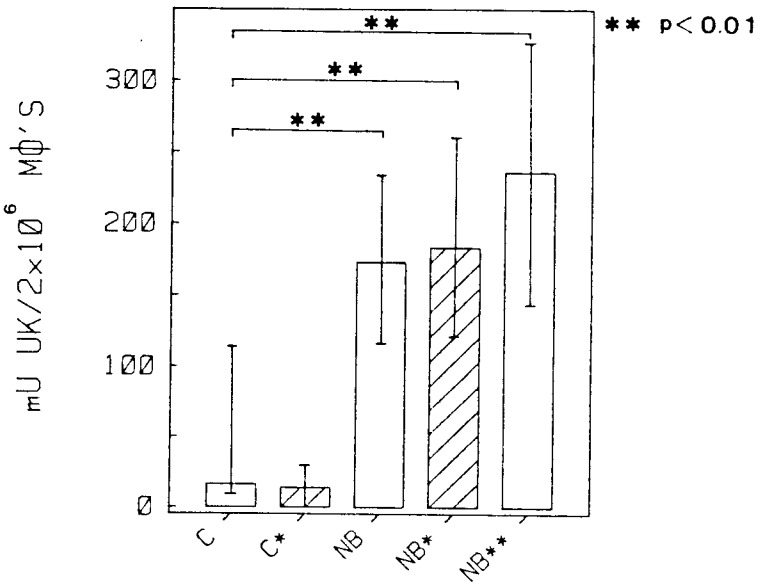


Fig 10 : Fibrinolysis produced by macrophages from mice inoculated 7 days previously with N.asteroides or with saline only : Direct assay

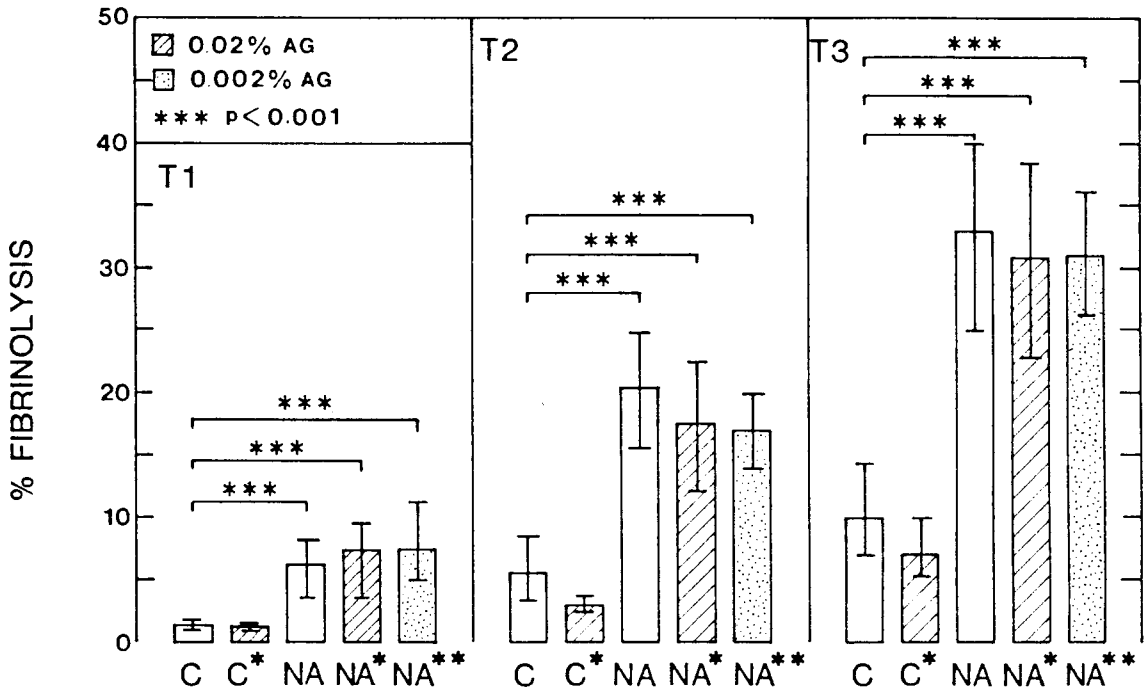


Fig 11 : Fibrinolysis produced by macrophages from mice inoculated 7 days previously with N.asteroides or with saline only : Indirect assay

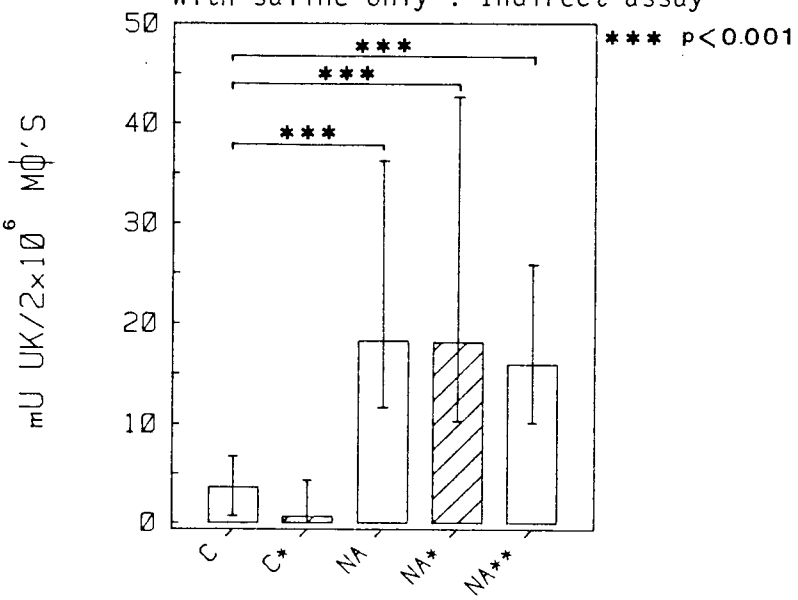


Fig 12 : Fibrinolysis produced by macrophages from mice inoculated 7 days previously with N. brasiliensis or with saline only : Direct assay

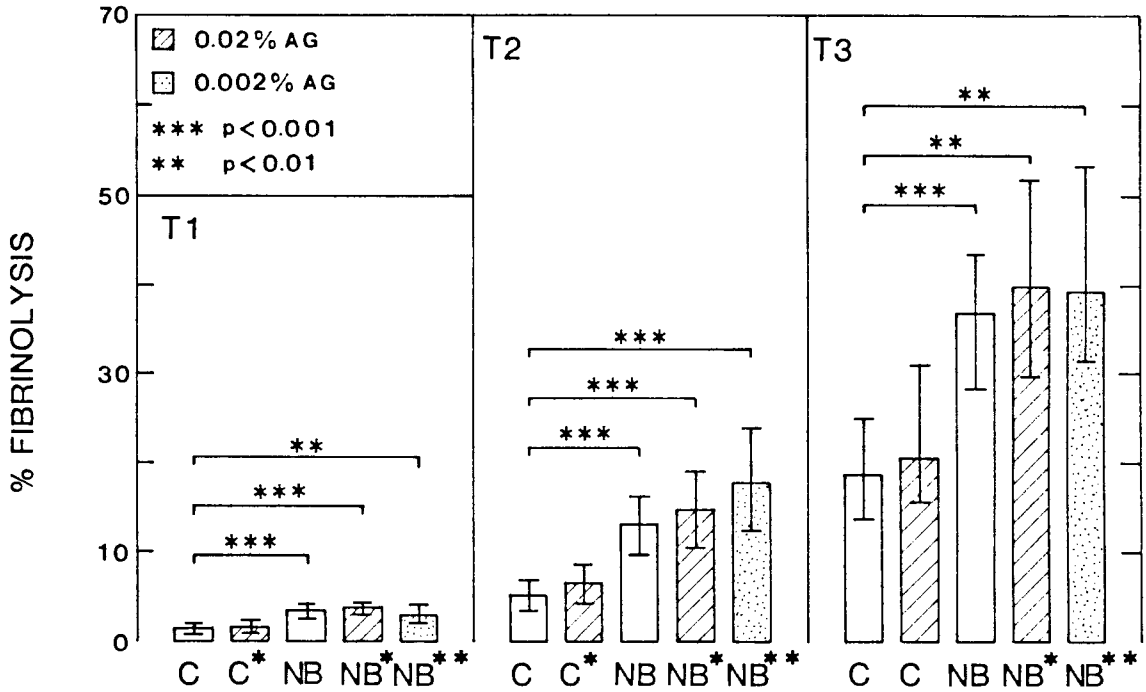


Fig 13 : Fibrinolysis produced by macrophages from mice inoculated 7 days previously with N.brasiliensis or with saline only : Indirect assay

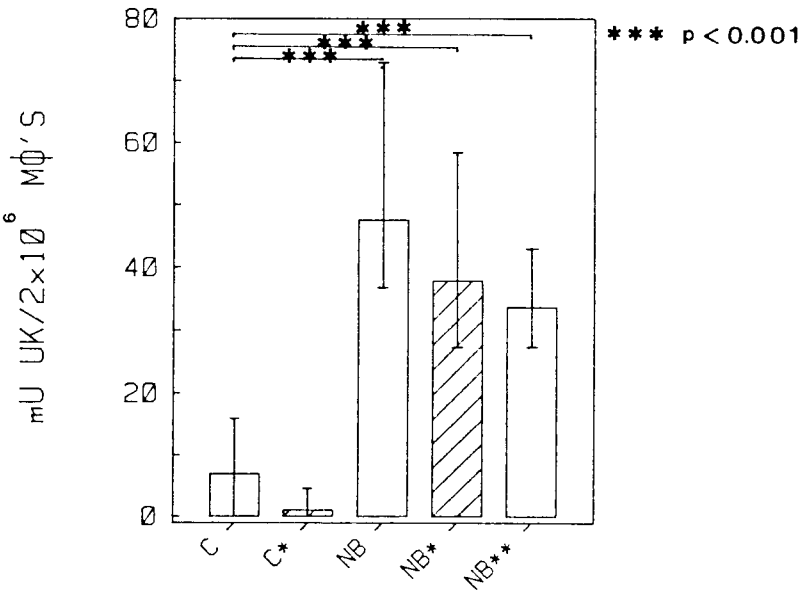


Fig 14 : Fibrinolysis produced by macrophages from mice inoculated 13 days previously with N.brasiliensis or with saline only : Direct assay

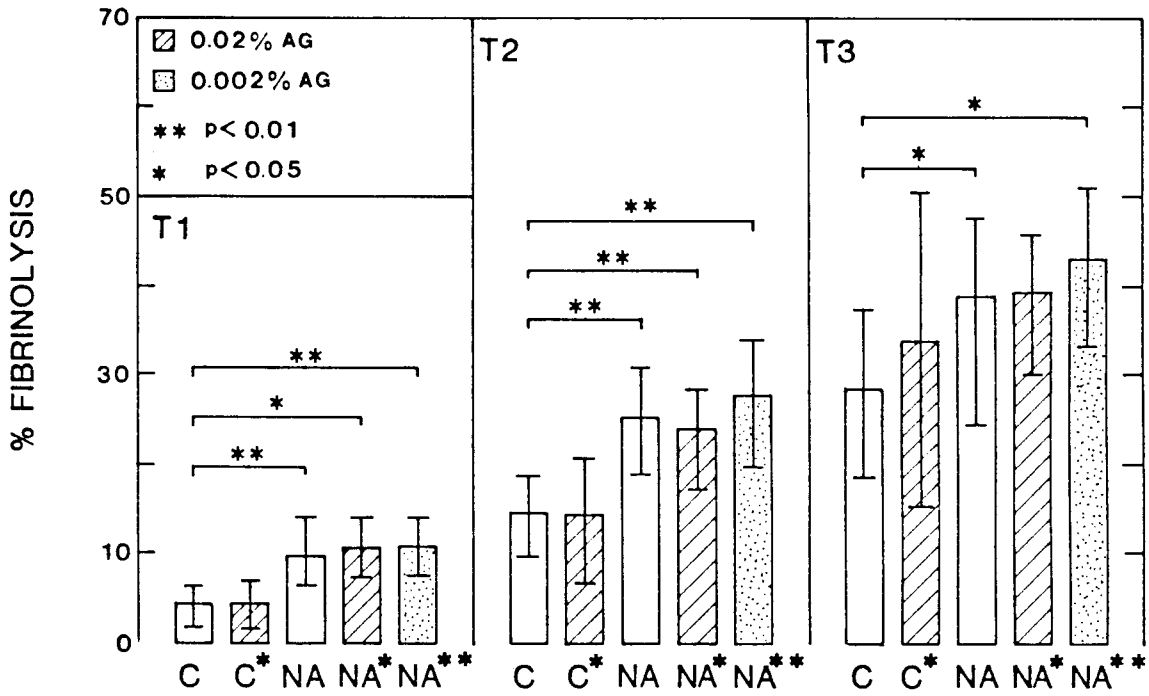


Fig 15 : Fibrinolysis produced by macrophages from mice inoculated 13 days previously with N.asteroides or with saline only : Indirect assay

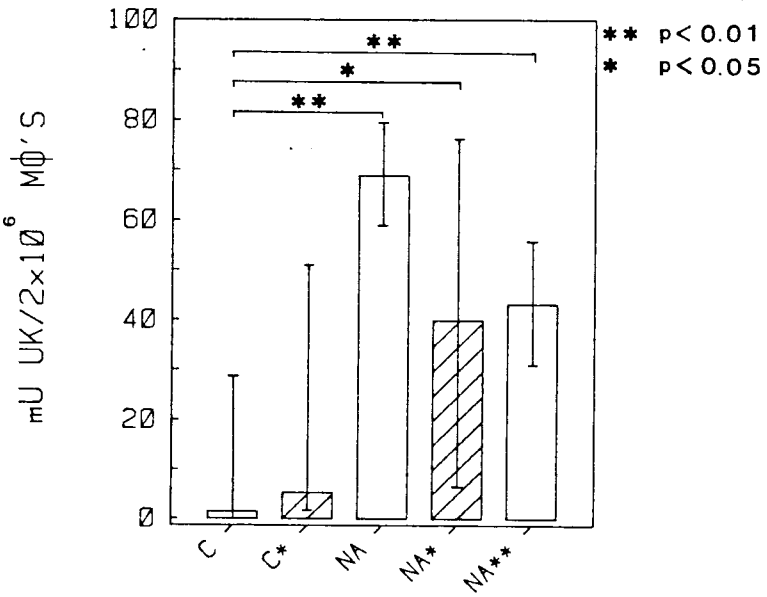


Fig 16 : Fibrinolysis produced by macrophages from mice inoculated 13 days previously with N.brasiliensis or with saline only : Direct assay

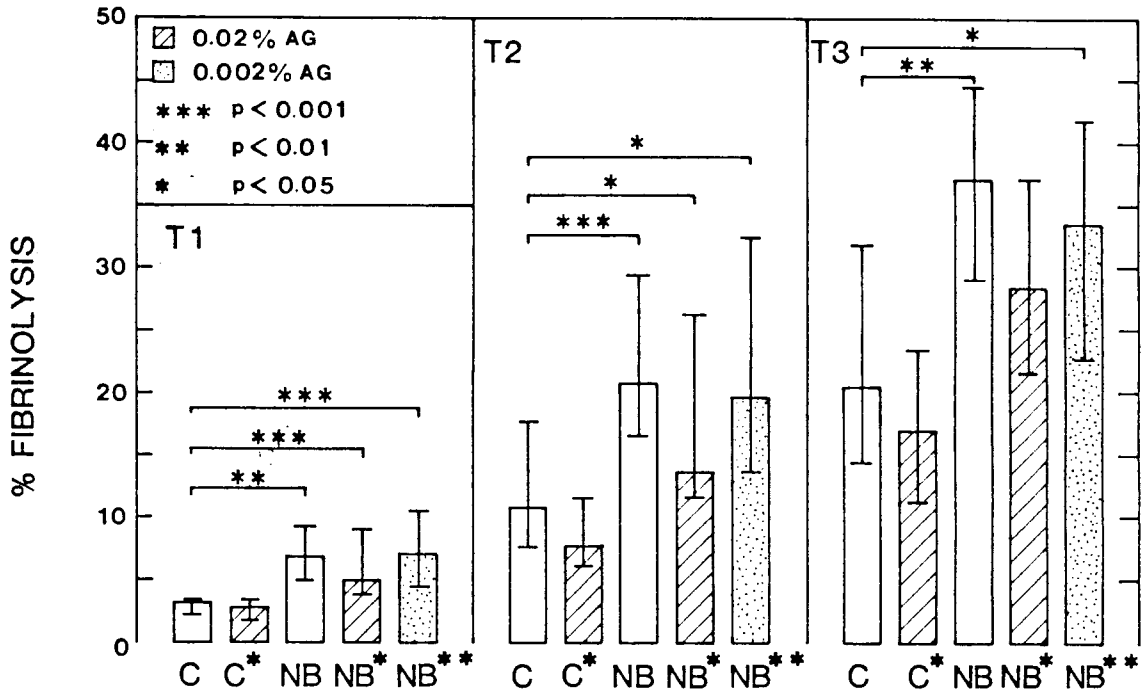


Fig 17. Fibrinolysis produced by macrophages from mice inoculated 13 days previously with N.brasiliensis or with saline only : Indirect assay

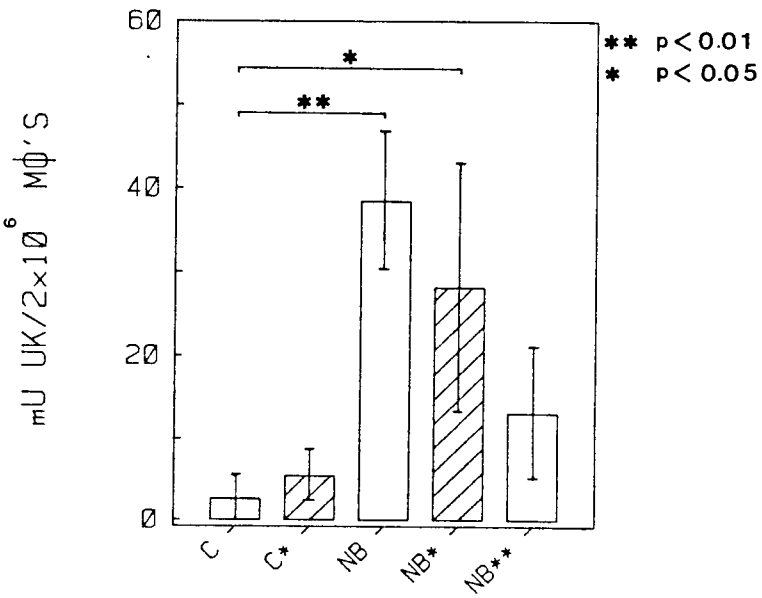


Fig 18 : Fibrinolysis produced by macrophages from mice inoculated 21 days previously with N.asteroides or with saline only : Direct assay

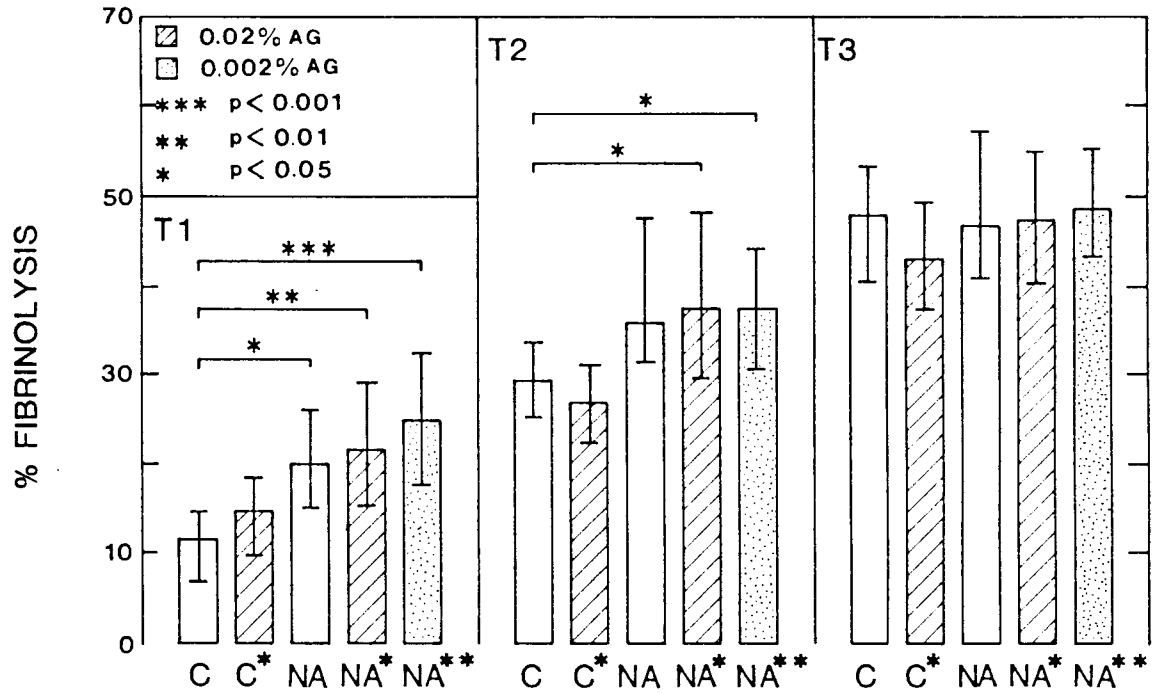


Fig 19 : Fibrinolysis produce d by macrophages from mice inoculated 21 days previously with N.asteroides or with saline only : Indirect assay

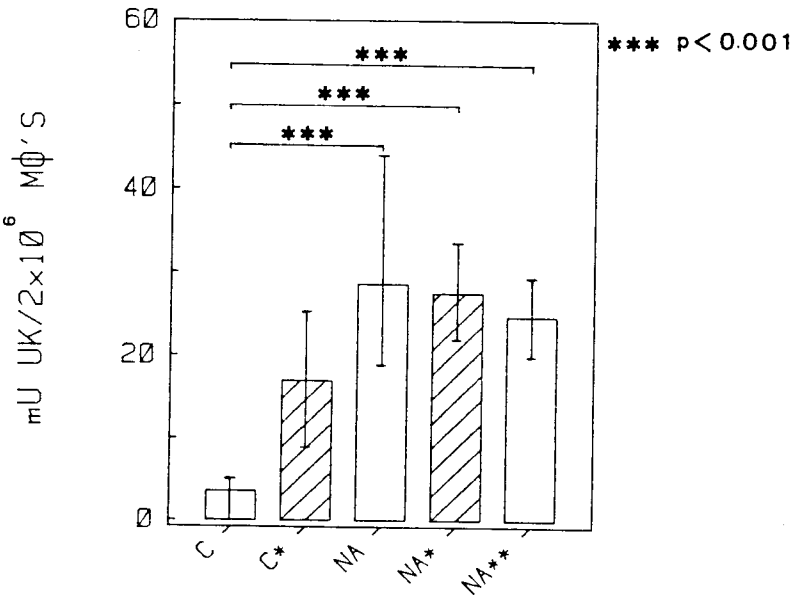


Fig 20 : Fibrinolysis produced by macrophages from mice inoculated 21 days previously with N.brasiliensis or with saline only : Direct assay

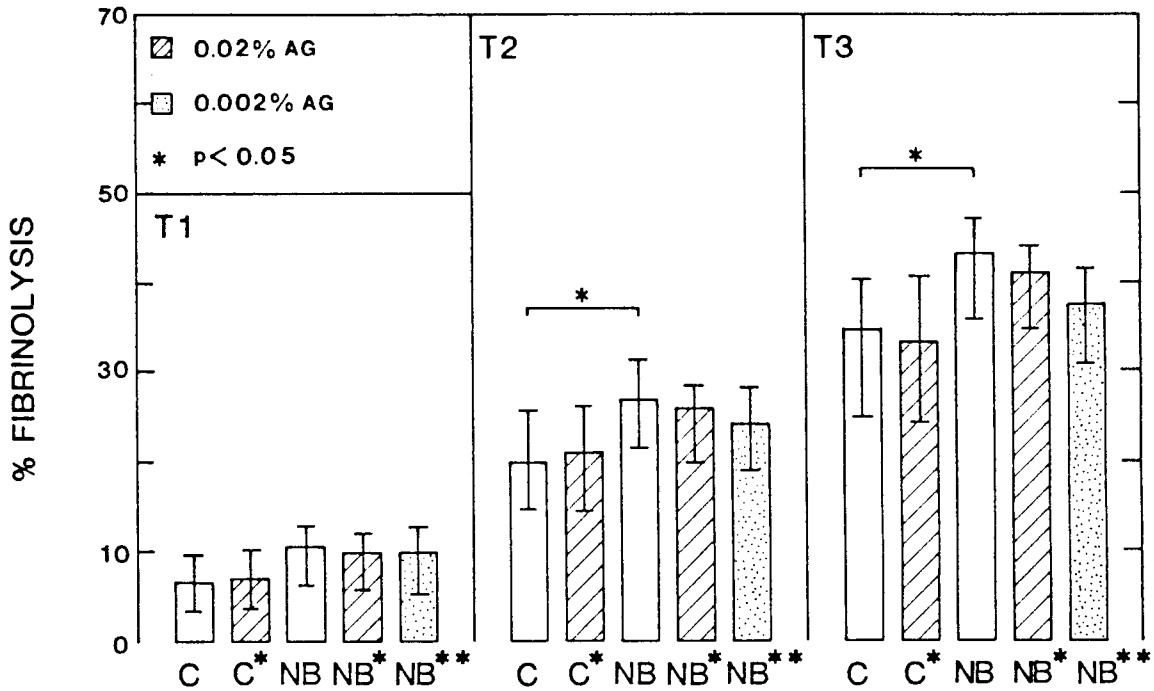
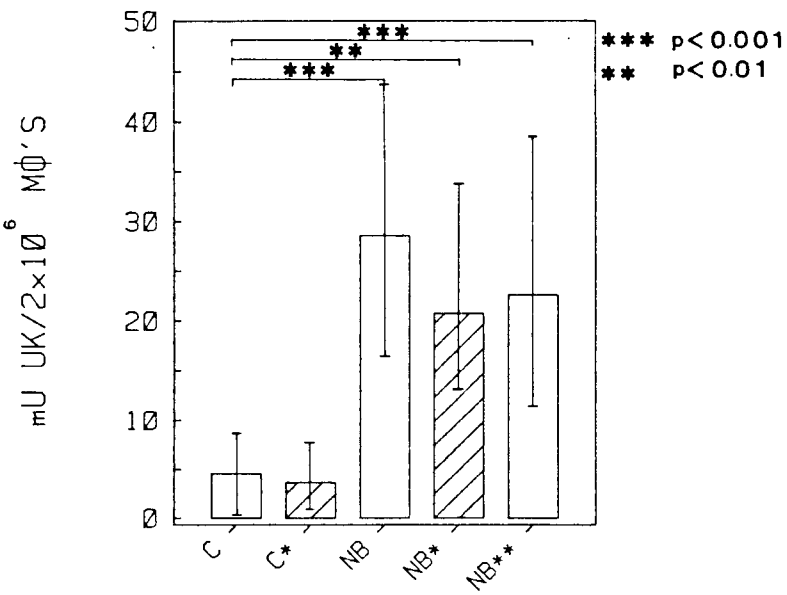


Fig 21 : Fibrinolysis produced by macrophages from mice inoculated 21 days previously with N.brasiliensis or with saline only : Indirect assay



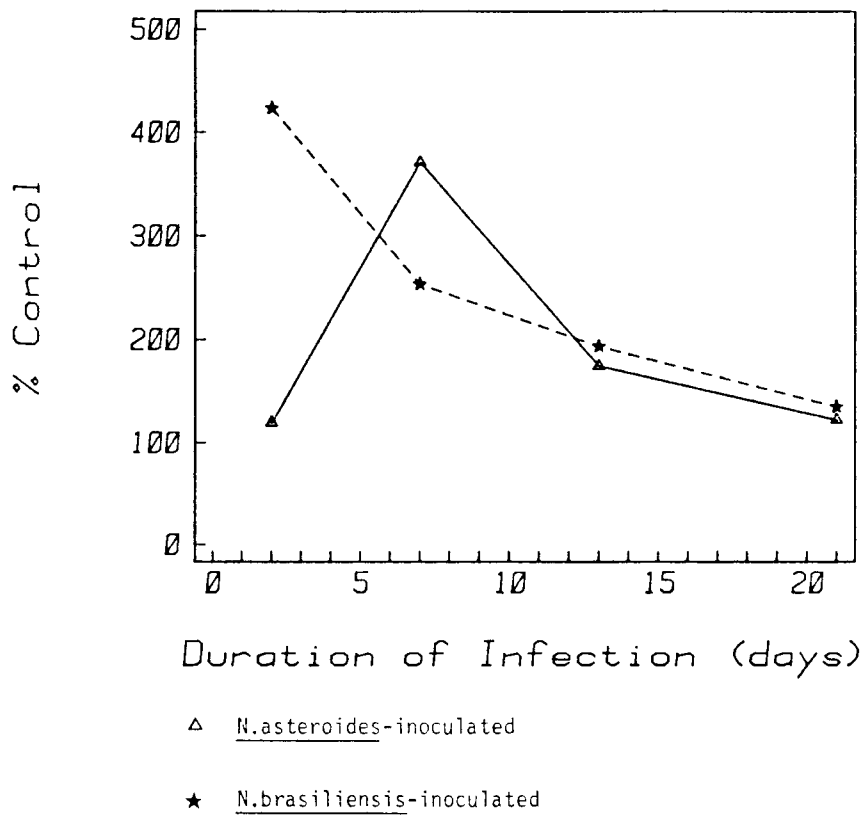


Fig 22 : Pattern of plasminogen activator secretion over the 21 day period of infection with N.asteroides and N.brasiliensis

3.6 TABLES

TABLE 12

Fibrinolysis produced by unstimulated macrophages exposed *in vitro* to Con A. (Data expressed as percentage fibrinolysis and are means of duplicates).

Mouse Strain	(Ha) ICR/UCT			
Timepoint (Hours)	T1 16	T2 20	T3 24	T4 42
Control	0.74	1.17	1.44	2.4
Control + 10^{-5}M Con A	1.18	1.38	1.68	2.49
Control + 10^{-6}M Con A	4.74	6.02	6.64	10.26
Control + 10^{-7}M Con A	5.01	8.23	11.42	14.57

KEY: M = Molarity

TABLE 13

Optimisation of number of macrophages distributed into each well : thioglycolate-elicited macrophages. (Data expressed as percentage fibrinolysis and are means of duplicates. ATHIFCS used as plasminogen source)

ATHIFCS	Mouse Strain	(Ha) ICR/UCT			
	Timepoint (Hours)	T1 5	T2 23	T3 28.25	T4 48
Control Cell no.	$5x10^5$	0.36	1.4	1.83	2.97
	1.25×10^5	0.7	3.92	4.96	6.4
	2.5×10^5	0.81	6.95	8.77	11.62
	5×10^5	1.2	13.28	16.28	20.36
	7.5×10^5	1.24	15.15	20.82	28.58
***	1×10^6	1.53	19.71	25.4	34.18
	1.25×10^6	1.0	15.09	19.65	25.85
	1.5×10^6	1.04	17.03	22.92	31.58
	1.75×10^6	1.3	21.45	27.58	28.23
	2×10^6	0.89	15.29	20.39	19.53

KEY: *** = Thioglycolate-elicited macrophages : cell number

TABLE 14

Optimisation of number of macrophages distributed into each well : mice inoculated 7 days previously with N.brasliensis (Data expressed as percentage fibrinolysis and are means of duplicates. ATDS used as plasminogen source)

ATDS	7 day N.brasiliensis infection : % fibrinolysis		
Timepoint (Hours)	T1 2	T2 4	T3 6
Control 5 x 10^5*	0.05	0.27	0.43
Nb 5 x 10^5	1.41	19.09	33.7
Nb x 1 x 10^6	10.78	54.61	72.88

* = macrophage number

TABLE 15a

Optimization of number of macrophages distributed into each well : mice inoculated 7 days previously with N.asteroides or N.brasiliensis. (Data expressed as percentage fibrinolysis and are means of duplicates. Purified plasminogen used as plasminogen source)

	PLG	7 day N.asteroides infection : % fibrinolysis		
	Timepoint (Hours)	T1 3.25	T2 4.5	T3 6
Control Cell no.	5 x 10^5	0.22	0.37	0.55
	2.5 x 10^5	1.55	8.93	16.2
Na	5 x 10^5	9.59	22.17	34.42
cell no.	7.5 x 10^5	24.23	31.28	45.66
	1 x 10^6	29.24	36.26	55.43
	1.5 x 10^6	22.3	37.44	59.28

TABLE 15b

	PLG	7 day N.brasiliensis infection : % fibrinolysis		
	Timepoint (Hours)	T1 2	T2 4.5	T3 6
Control Cell no.	5 x 10^5	1.15	3.74	7.1
	2.5 x 10^5	2.55	4.81	10.84
Nb cell no.	5 x 10^5	4.41	14.43	29.92
	7.5 x 10^5	7.61	21.66	36.72

TABLE 16a

Optimisation of volume of conditioned medium (CM) to be added to each well and period of culture in serum-free medium : 21 day N.asteroides infection, 24 hour incubation. (Data expressed as percentage fibrinolysis are means of duplicates)

	24 Hour incubation	21 day N.asteroides infection : % fibrinolysis		
	Timepoint (Hours)	T1 1	T2 4	T3 10
	Volume:			
Volume of CM from control macrophages	20 µl	0.13	0.94	6.75
	50 µl	0.15	1.45	8.96
	100 µl	0.27	2.68	7.48
	200 µl	0.16	3.24	16.4
	250 µl	0.02	0.37	8.43
Volume of CM from Na	20 µl	0.44	3.99	31.74
	50 µl	0.71	8.78	50.33
	100 µl	0.53	8.68	62.08
	200 µl	0.49	8.1	62.48
	250 µl	0.49	8.22	55.52

TABLE 16b

Optimisation of period of culture in serum-free medium : 21 day N.asteroides infection, 4 days incubation. (Data expressed as percentage fibrinolysis and are means of duplicates).

	4 day incubation	21 day N.asteroides infection : % fibrinolysis		
	Timepoint (Hours)	T1 1	T2 4	T3 10
	Volume:			
Control CM	50 µl	0	0	3.14
Na CM	50 µl	2.12	13.49	96.84

TABLE 16c

Optimisation of volume of CM to be added to each well : 2 day N.brasiliensis infection, 24 hour incubation in serum-free medium. (Data expressed as % fibrinolysis and are means of duplicate)

	24 Hour incubation	21 day N.brasiliensis infection : % fibrinolysis		
	Timepoint (Hours)	T1 3.5	T2 7.5	T3 18.5
	Volume:			
Volume of CM from control macrophages	20 µl	0.38	1.21	5.52
	50 µl	0.86	0.51	7.72
	100 µl	1.02	2.35	16.92
Volume of CM from Nb	20 µl	4.36	24.47	63.97
	50 µl	9.27	62.6	68.91
	100 µl	18.44	81.06	78.47

TABLE 17a

Comparison of macrophage fibrinolysis in dog serum and in fetal calf serum. (Data expressed as % fibrinolysis and are means of duplicates)

		% Fibrinolysis						
	Timepoint (Hours)	T1 1.25	T2 3.5	T3 7.25	T4 11.5	T5 22	T6 30.25	T7 54.25
ATHI FCS	Control	0.25	0.28	0.35	0.65	0.86	1.7	3.84
	Thio	1.06	1.34	2.05	4.56	11.37	17.23	28.52
ATDS	Control	0.25	6.45	39.86	62.29			
	Thio	12.82	40.89	61.51	56.2			
	2d Na	0.05	3.26	18.03	30.97			

TABLE 17b

Comparison of macrophage fibrinolysis in dog serum and in fetal calf serum. (Data expressed as % fibrinolysis and are means of duplicates) Mice inoculated 7 days previously with N.brasiliensis.

		% Fibrinolysis						
	Timepoint (Hours)	T1 1.75	T2 4	T3 6	T4 12.25	T5 22.25	T6 30	T7 51.25
ATFCS	Control	0.1	0	0	0	0.1	0	0
	7d Nb	0.1	0.03	0.23	1.31	3.76	4.01	6.3
ATDS	Control	0.05	0.27	0.43	1.91	10.26		
	7d Nb	1.41	19.09	33.7	78.82	86.59		
ATFCS -P	Control	0	0	0	0	0	0	0
	7d Nb	0	0	0	0	0	0	0

TABLE 18

Fibrinolysis by control macrophages from individual mice in different batches of ATDS. (Data expressed as % fibrinolysis and are means of duplicates)

Mouse No.	ATDS Batch	Control macrophages from individual mice : % fibrinolysis		
	Timepoint (Hours)	T1 2	T2 4.25	T3 6
1	C	5.31	22.25	38.22
1	N	9.66	30.87	47.77
2	N	5.0	42.36	56.95
3	C	8.27	30.83	43.28
3	N	10.56	33.46	57.94
3	H	10.07	21.6	44.15
4	N	5.75	29.33	54.05
5	N	13.87	50.95	69.01
6	H	3.21	24.05	48.16
8	N	4.51	21.93	39.27

KEY: C = Conventionally used batch of ATDS
N = New batch of ATDS
H = Home prepared ATDS

TABLE 19

Plasminogen-dependence of macrophage fibrinolysis in ATDS. Macrophages from mice inoculated 13 days previously with N.brasiliensis. (Data expressed as % fibrinolysis and are means of duplicates)

		13 day N.brasiliensis infection : % Fibrinolysis			
	Timepoint (Hours)	T1 2	T2 6	T3 19	T4 25.5
Control	ATDS	1.27	5.72	48.74	75.18
	ATDS -P	Ø	Ø	Ø	Ø
Na	ATDS	Ø.16	2.63	26.34	50.5
	ATDS -P	Ø	Ø	Ø	Ø

KEY: ATDS -P = Acid-treated dog serum depleted of plasminogen

TABLE 2Ø

Fibrinolysis produced by macrophages from individual control mice in HIATFCS and ATDS. (Data expressed as % fibrinolysis and are means of duplicates)

Serum	Mouse No.	Timepoint (Hours)			
		T1 2.25	T2 4	T3 6	T4 22
HIAT FCS	3	Ø.32	Ø.35	Ø.21	Ø
	5	Ø.12	Ø	Ø	Ø
	12	Ø.18	Ø.36	Ø.32	Ø.51
ATDS	1	7.22	16.86	29.63	8Ø.92
	2	4.14	11.48	28.26	87.92
	4	3.46	7.84	17.2	86.32
	6	13.68	18.79	37.36	8Ø.87
	7	3.89	8.11	25.11	8Ø.99
	8	2.91	6.Ø1	16.14	78.16
	9	4.77	1Ø.23	28.15	84.82
	1Ø	1.26	5.4	13.59	73.61
	12	9.78	23.79	39.51	86.18
	13	2.71	5.76	13.67	63.7
	14	1.18	2.27	5.37	26.64
	15	4.98	8.78	18.81	7Ø.21
	16	3.31	6.53	13.95	59.68
	18	3.72	1Ø.11	2Ø.53	72.95
	19	4.58	13.53	26.12	83.29
	2Ø	2.37	5.27	15.84	67.62

TABLE 21

Effect of removal of lymphocytes on macrophage fibrinolysis in ATDS. Macrophages from mice inoculated 7 days previously with N.asteroides. (Data expressed as % fibrinolysis and are means of duplicates)

MØ Type	Presence or absence of lymphocytes	Timepoint (Hours)			
		T1 3	T2 5.5	T3 7	T4 11.5
Control	+ L	8.31	47.97	59.61	60.09
	- L	7.23	46.58	60.86	73.81
7d Na	+ L	11.41	30.63	47.95	51.61
	- L	8.84	30.66	46.35	49.18

TABLE 22

Comparison of macrophage fibrinolysis in the presence of purified plasminogen (PLG) or ATDS and confirmation of plasminogen dependence of fibrinolysis. Macrophages from mice inoculated 7days previously with N.brasiliensis. (Data expressed as percentage fibrinolysis and are means of duplicates)

MØ Type	PLG Source	Timepoint (Hours)		
		T1 2.5	T2 4.5	T3 8
Control	PLG	2.83	10	32.03
	ATDS	13.99	44.81	71.94
	- PLG	Ø	Ø	Ø
7d Nb	PLG	3.18	19.94	52.88
	ATDS	19.27	33.79	52.33
	- PLG	Ø	Ø	Ø

KEY: 7d Na = Mice inoculated 7 days previously with N.asteroides
7d Nb = Mice inoculated 7 days previously with N.brasiliensis

TABLE 23a

Comparison of macrophages fibrinolytic activity when measured by the direct and indirect assay. Mice were inoculated 7 days previously with N.brasiliensis, and macrophages assayed by the two methods were derived from the same cell pool. (Data expressed as median % fibrinolysis and 95% confidence limits for n = 8 replicates [direct assay] and n = 4 replicates [indirect assay])

MØ Type	Direct Assay	Timepoint (Hours)		
		T1 2	T2 4	T3 6
Control	n	8	8	8
	Median	1.72	6.45	24.82
	LL	1.51	5.05	21.65
	UL	1.99	7.87	28.58
7d Nb	n	8	8	8
	Median	3.84	14.28	41.34
	LL	2.61	9.94	37.04
	UL	4.71	19.24	46.57

TABLE 23b

Fibrinolysis measured by the indirect assay

MØ Type	Indirect Assay	Timepoint (Hours)		
		T1 6.75	T2 8.25	T3 10.75
Control	n	4	4	4
	Median	Ø	Ø	Ø
	LL	Ø	Ø	Ø
	UL	Ø	Ø	Ø
7d Nb	n	4	4	4
	Median	7.43	11.24	26.76
	LL	6.14	9.69	23.19
	UL	9.71	12.79	30.33

KEY: 7d Nb = mice inoculated 7 days previously with N.brasiliensis

TABLE 24a

Effect of Con A exposure *in vitro* on fibrinolysis by thioglycolate-elicited peritoneal macrophages, and selection of appropriate mouse strain. (Units = % fibrinolysis data : mean of duplicates)

Mouse Strain	(Ha) ICR/UCT				C_3H/He/UCT			
Timepoint (Hours)	T1 5.5	T2 21.5	T3 27.5	T4 42	T1 5.5	T2 21.5	T3 27.5	T4 42
Control	1.19	2.24	2.47	3.83	Ø.6	1.32	1.59	2.19
Thio	1.85	8.22	10.08	17.41	1.63	14.86	20.52	38.2
Thio (a)	1.79	7.98	9.85	16.59	1.43	8.03	10.64	17.67
Thio (b)	1.89	9.98	13.04	22.79	1.87	17.25	23.34	39.27
Thio (c)	1.72	8.65	10.73	17.53	1.73	15.88	19.4	31.69

TABLE 24b

Mouse Strain	Balb/C/UCT				CBA/Ca/UCT			
Timepoint (Hours)	T1 5.5	T2 21.5	T3 27.5	T4 42	T1 5.5	T2 21.5	T3 27.5	T4 42
Control	Ø.16	1.45	1.77	2.51	1.34	5.11	4.92	7.53
Thio	1.24	4.11	5.Ø5	7.3	1.74	5.37	6.Ø5	8.56
Thio (a)	1.Ø4	4.15	4.1	7.23	1.14	2.58	3.Ø9	4.94
Thio (b)	1.96	9.82	12.31	18.66	1.41	4.93	5.97	9.15
Thio (c)	1.Ø2	5.93	6.96	1Ø.74	1.34	4.73	5.69	8.65

KEY: Thio (a) = Thio, $1Ø^{-6}$M Con A
Thio (b) = Thio, $1Ø^{-7}$M Con A
Thio (c) = Thio, $1Ø^{-8}$M Con A

TABLE 25

Fibrinolysis by macrophages over the 48 hour incubation period prior to the assay for fibrinolysis. Macrophages are from mice inoculated 7 days previously with *N.brasiliensis*. (Data expressed as median % fibrinolysis for n = 8 replicates and 95% confidence limits)

MØ Type	% Fibrinolysis by Macrophages during the 48 hour incubation period		
	n	Median	LL	UL
Control	8	4.81	4.Ø7	5.59
Nb	8	9.54	8.74	1Ø.31
Nb + Ø.Ø2%	8	1Ø.57	9.36	11.87
Nb + Ø.ØØ2%	8	1Ø.93	1Ø.36	11.46

KEY: Control = Macrophages from saline-inoculated mice
Nb = Macrophages from mice inoculated 7 days previously with *N.brasiliensis*
Nb + Ø.Ø2% (Ø.ØØ2%) = Macrophages from mice inoculated 7 days previously with *N.brasiliensis* and exposed *in vitro* to Ø.Ø2% (Ø.ØØ2%) *N.brasiliensis* antigen

TABLE 26

Macrophage counts in washes after 48 hour incubation period. (Data expressed as number of macrophages counted in washes from individual linbro wells, using a haemocytometer. N + 0.02% and N + 0.002% = Nocardia-activated macrophages exposed in vitro to 0.02% or 0.002% homologous antigen)

	Macrophage counts in washes after 48 hour incubation				
	E	C	N	N+0.02%	N+0.002%
Na	1	1	1	1	2
	2	1	2	0	1
	3	2	0	2	1
Nb	4	0	1	1	0
	5	1	0	1	1
	6	0	2	1	0

KEY: E = Experiment
C = Control
N = Nocardia-activated
Na = N.asteroides infection
Nb = N.brasiliensis infection

TABLE 27

Cell counts after fibrinolysis assay. Adherent cells were treated with versene solution and cell counts determined using a coulter counter. Macrophages were harvested from mice inoculated 21 days previously with N.asteroides (Na + 0.02% or 0.002% = N.asteroides -activated macrophages exposed in vitro to 0.02% or 0.002% N.asteroides antigen)

21d Na	Cell counts after assay	
Macrophage type	Mean Count	Total Cells
Control	2235	89400
	1961	78440
Na	2047	81880
	3420	136800
Na + 0.02%	1666	66640
Na + 0.002%	2701	108040

KEY: 21d Na = mice inoculated 21 days previously with N.asteroides

TABLE 28a

Dose response and time response data : 7 day N.asteroides infection. Macrophages were incubated with or without various concentrations of N.asteroides antigen for 24 or 48 hours and fibrinolysis measured. (Data expressed as % fibrinolysis and are means of duplicates)

MØ Type	24 Hr Incubation	Timepoint (Hours)		
	Ag conc	T1 2	T2 4	T3 6
C	-	0.44	0.53	0.9
	0.002%	0.46	0.6	0.82
Na	-	1.54	2.7	7.98
	0.2%	1.31	3.5	11.9
	0.02%	1.09	3.0	12.12
	0.002%	0.98	2.28	6.14
	0.0002%	1.01	2.13	7.77
	0.00002%	1.29	2.71	10.36

TABLE 28b

MØ Type	48 Hr Incubation	Timepoint (Hours)		
	Ag conc	T1 2	T2 4	T3 6
C	-	Ø.22	Ø.37	Ø.55
	Ø.ØØ2%	Ø.16	Ø.41	Ø.73
Na	-	9.59	22.17	34.42
	Ø.2%	1Ø.38	2Ø.32	35.88
	Ø.Ø2%	15.7	25.61	41.51
	Ø.ØØ2%	16.57	28.15	44.39
	Ø.ØØØ2%	11.21	21.64	33.45
	Ø.ØØØØ2%	14.16	22.71	37.41

TABLE 29a

Dose response and time response data : 7 day N.asteroides infection. Macrophages were incubated with or without various concentrations of N.asteroides antigen for 48 or 72 hours and fibrinolysis measured. (Data expressed as % fibrinolysis and are means of duplicates)

MØ Type	48 Hr Incubation	Timepoint (Hours)		
	Ag conc	T1 2	T2 4	T3 6
C	-	1.89	6.Ø8	11.32
	Ø.ØØ2%	Ø.65	1.81	4.1
Na	-	4.92	1Ø.68	19.83
	Ø.2%	1.52	3.69	7.65
	Ø.Ø2%	3.15	5.77	11.77
	Ø.ØØ2%	2.68	9.22	17.Ø4
	Ø.ØØØ2%	3.58	7.97	16.79
	Ø.ØØØØ2%	4.65	8.62	17.76

TABLE 29b

MØ Type	72 Hr Incubation	Timepoint (Hours)		
	Ag conc	T1 2	T2 4	T3 6
C	-	Ø.68	5.54	19.79
	Ø.ØØ2%	Ø.29	2.Ø4	8.69
Na	-	Ø.5	5.51	14.88
	Ø.2%	Ø.19	1.27	4.76
	Ø.Ø2%	Ø.Ø1	2.21	7.14
	Ø.ØØ2%	Ø.48	3.72	11.56
	Ø.ØØØ2%	Ø.68	3.7	13.49
	Ø.ØØØØ2%	Ø.25	4.23	15.17

TABLE 30a

Dose response and time response data : 13 day N.brasiliensis infection. Macrophages were incubated with or without various concentrations of N.brasiliensis antigen for 48 or 72 hours and fibrinolysis measured. (Data expressed as % fibrinolysis and are means of duplicates)

MØ Type	48 Hr Incubation	Timepoint (Hours)		
	Ag conc	T1 2	T2 4	T3 6
C	-	Ø.86	5.Ø5	9.45
	Ø.ØØ2%	1.19	6.33	1Ø.44
Nb	-	2.92	15.33	23.9
	Ø.2%	3.65	19.9	3Ø.65
	Ø.Ø2%	3.73	12.95	25.29
	Ø.ØØ2%	2.58	1Ø.62	2Ø.Ø5
	Ø.ØØØ2%	2.81	16.79	26.29
	Ø.ØØØØ2%	1.7	6.35	12.76

TABLE 3Øb

MØ Type	72 Hr Incubation	Timepoint (Hours)		
	Ag conc	T1 2	T2 4	T3 6
C	-	Ø.36	Ø.62	1.64
	Ø.ØØ2%	Ø.39	1.45	3.Ø
Nb	-	5.Ø3	21.26	49.75
	Ø.2%	3.71	17.73	46.94
	Ø.Ø2%	5.8	21.2	56.55
	Ø.ØØ2%	4.56	19.78	52.89
	Ø.ØØØ2%	3.9	15.63	52.21
	Ø.ØØØØ2%	5.1	19.56	52.89

TABLE 31

Fibrinolysis produced by macrophages from mice inoculated 2 days previously with N.asteroides or saline only. Direct assay. % Fibrinolysis measured at 2 hourly intervals over 6 hours.

A	E	T1 2 Hr % fibrinolysis		T2 4Hr % fibrinolysis		T3 6Hr % fibrinolysis	
C	1	0.62	0.19	2.03	1.73	4.79	2.78
	2	6.98	19.31	28.73	37.05	60.07	74.47
		9.7	15.51	27.39	35.71	57.56	68.86
		18.93	12.51	38.10	32.03	66.59	71.34
		7.61	12.63	35.91	37.3	66.87	64.13
	3	52.22	21.71	80.21	64.31	77.15	80.94
		36.27	66.79	79.72	83.12	75.62	83.15
		51.67	42.28	76.08	79.81	75.91	90.38
		41.49	56.22	72.91	84.52	80.35	87.8
C*	1	0.86	0.76	3.27	3.51	6.14	6.62
	2	14.11	18.95	30.73	28.4	54.94	62.6
		12.01	15.65	27.11	42.28	59.08	66.7
		8.89	18.78	24.06	40.52	54.82	75.39
		11.98	12.66	24.71	33.47	57.73	65.52
	3	29.64	21.72	64.26	70.14	73.66	86.39
		29.31	41.2	71.28	74.89	80.76	78.79
		16.47	55.87	71.64	76.44	70.69	77.2
		38.22	31.61	79.33	69.87	78.57	74.77
Na	1	2.21	2.21	5.98	5.75	14.71	10.59
	2	29.03	14.17	55.48	65.11	82.27	90.19
		21.26	17.26	56.58	61.51	82.96	87.17
		15.34	30.92	51.57	55.67	84.53	79.71
		18.48	22.02	54.91	59.6	85.18	81.55
	3	57.96	43.13	70.32	74.51	71.15	78.64
		39.26	45.75	76.41	82.36	71.86	77.2
		36.88	54.15	77.85	72.93	74.55	72.95
		49.12	43.48	71.34	76.73	78.62	77.08
Na*	1	3.89	2.98	9.04	7.12	16.36	14.12
	2	21.66	9.94	47.53	25.57	75.2	52
		19.96	9.64	55.24	25.17	74.77	50.35
		19.59	8.48	58.69	24.92	79.82	48.49
		16.54	7.34	57.75	20.38	76.76	46.96
	3	41.55	43.42	74.8	69.57	70.41	71.14
		43.25	48.71	89.11	72.17	71.17	71.83
		48.02	64.07	73.81	74.01	73.68	75.49
		44.0	50.77	79.85	75.82	72.57	77.43
Na**	1	0.3	0.67	1.73	2.79	3.84	5.58
	2	18.07	6.88	33.75	18.41	60.9	44.68
		19.9	11.07	39.35	29.42	74.11	47.6
		11.66	11.4	28.73	25.79	57.15	52.17
		8.14	12.29	21.01	35.61	50.4	64.61
	3	40.08	29.31	68.05	72.62	69.04	76.78
		54.34	77.26	68.62	78.43	70.64	77.42
		45.05	32.19	71.67	84.34	75.41	78.88
		41.69	47.22	64.73	82.9	73.86	76.84

KEY:
- A = 2d N.asteroides direct assay
- E = Experiment number
- C = Control macrophages
- C* = Control macrophages + 0.02% Antigen in vitro
- Na = N.asteroides-activated macrophages
- Na* = N.asteroides-activated macrophages + 0.02% Antigen in vitro
- Na** = N.asteroides-activated macrophage + 0.002% Antigen in vitro

TABLE 32

Two day N.asteroides infection : Median % fibrinolysis values and their 95% confidence limits.

2d Na Direct		T1		T2		T3	
Control	M	n=18	25.99	n=18	53.555	n=18	71.34
	LL		12.955		34.54		62.08
	UL		36.885		60.61		77.71
Control + 0.02% Ag	M	n=18	19.595	n=18	48.49	n=18	66.93
	LL		13.815		32.29		56.34
	UL		28.365		58.585		74.53
Na	M	n=18	30.085	n=18	64.15	n=18	78.2
	LL		20.735		54.075		72.41
	UL		39.91		70.83		81.91
Na + 0.02% Ag	M	n=18	27.33	n=18	50.37	n=18	63.56
	LL		14.765		40.08		49.42
	UL		40.305		68.32		73.97
Na + 0.002% Ag	M	n=18	24.915	n=18	47.075	n=18	62.76
	LL		12.295		29.77		48.43
	UL		37.12		59.255		72.23

TABLE 33

Indirect assay data for macrophages from mice inoculated 2 days previously with N.asteroides or normal saline. Results expressed as milliunits of urokinase and as specific activity.

Indirect Assay	Macrophage type					
	E	C	C*	Na	Na*	Na**
(a)	1	6.29	8.43	15.91	11.48	
	2			16.47	15.83	25.16
	3	16.35	5.3	32.56	23.18	15.03
		7.57				6.42
		7.65		43.38	44.54	25.26
(b)	1	30.1	45.62	82.4	79.61	
	2			147.54	88.76	269.9
	3	68.13	13.96	153.67	65.89	49.93
		24.43				32.72
		30.55		216.29	211.45	122.93

KEY: E = Experiment
C = Control macrophages
C* = Control macrophages + 0.02% antigen in vitro
Na = N.asteroides-activated macrophages
Na* = N.asteroides-activated macrophages + 0.02% antigen in vitro
Na** = N.asteroides-activated macrophages + 0.002% antigen in vitro
(a) = Total milliunits of urokinase (mU Uk)
(b) = Specific activity (mU Uk/mg protein)

TABLE 34

Two day N.asteroides infection : Median values and their 95% confidence limits for indirect assay. Data expressed as milliunits of urokinase and as specific activity.

Indirect Assay		Macrophage type				
		C	C*	Na	Na*	Na**
(a)		n=4	n=2	n=4	n=4	n=4
	M	7.63	5.3	27.08	21.34	17.97
	LL	4.02	-4.64	17.23	9.49	11.22
	UL	11.24	15.24	36.93	33.19	24.72
(b)		n=4	n=2	n=4	n=4	n=4
	M	30.44	13.96	149.98	86.47	104.68
	LL	14.77	-86.61	131.99	34.47	19.65
	UL	46.11	114.53	197.98	138.65	189.7

KEY: C = Control macrophages
C* = Control macrophages + 0.02% antigen in vitro
Na = N.asteroides-activated macrophages
Na* = N.asteroides-activated macrophages + 0.02% antigen in vitro
Na** = N.asteroides-activated macrophages + 0.002% antigen in vitro
(a) = Total milliunits of urokinase (mU Uk)
(b) = Specific activity (mU Uk/mg cell protein)

TABLE 35

Significance level (Mann Whitney U Test, 2 tailed) achieved on comparison of different experimental data groups : 2 day N.asteroides infection.

	Direct Assay			Indirect Assay	
Data groups compared	T1 2Hr	T2 4Hr	T3 6Hr	Milliunits of urokinase	Specific Activity
1,2					
1,3				5%	5%
1,4				5%	5%
1,5					
2,3					5%
2,4					5%
2,5					
3,4			1%		
3,5			1%		
4,5					

KEY: 1 = Control macrophages
2 = Control macrophages + 0.02% antigen
3 = N.asteroides-activated macrophages
4 = N.asteroides-activated macrophages + 0.02% antigen in vitro
5 = N.asteroides-activated macrophages + 0.002% antigen in vitro
* = Borderline significance

TABLE 36

Fibrinolysis produced by macrophages from mice inoculated 2 days previously with N.brasiliensis or saline only. Direct assay % fibrinolysis measured at 2 hourly intervals over 6 hours.

A	T1 2 Hr % fibrinolysis		T2 4Hr % fibrinolysis		T3 6Hr % fibrinolysis	
C	Ø.93	1.18	7.36	9.Ø8	3Ø.1	3Ø.95
	1.Ø4	1.34	4.15	5.13	12.86	17.54
	5.82	7.Ø4	32.Ø6	32.15	51.25	51.22
	Ø.53	1.28	3.85	4.56	14.22	18.37
	2.87	2.Ø1	5.38	5.Ø5	9.38	8.62
	2.75	3.94	6.23	5.65	11.93	9.79
	3.16	4.6	5.95	8.34	1Ø.76	14.69
	5.12	2.9	13.75	4.75	18.74	9.45
C*	1.68	1.33	8.71	6.3	3Ø.5	22.8
	1.Ø	Ø.97	4.Ø2	3.36	11.68	1Ø.34
	Ø.37	Ø.52	2.95	3.Ø3	11.58	12.21
	2.93	2.58	5.19	6.9	1Ø.1	11.16
	2.81	3.78	5.75	8.8	13.9	11.73
	2.3	4.55	6.Ø7	9.66	9.23	6.18
	3.68	4.17	5.12	9.18	8.62	13.77
Nb	3.64	3.4	24.Ø8	19.39	55.18	48.97
	1.73	1.Ø4	3.8	3.3	8.91	9.Ø3
	15.67	11.Ø8	46.45	38.34	6Ø.19	58.28
	1.48	1.95	5.69	7.33	17.15	24.15
	11.14	23.52	29.24	28.99	42.34	46.56
	13.49	3Ø.13	636.49	48.56	45.98	74.5
	15.24	2Ø.4	32.53	43.26	48.44	6Ø.15
	16.1	19.57	19.35	52.25	26.62	69.25
Nb*	1.8	4.43	17.Ø2	33.63	4Ø.67	52.67
	1.59	1.66	4.13	5.77	15.75	15.95
	11.Ø8	9.28	41.65	54.18	58.97	57.62
	1.8	1.Ø2	6.63	3.52	22.Ø5	1Ø.56
	11.Ø6	2Ø.44	31.77	39.79	46.91	56.63
	24.69	18.84	44.61	38.62	49.55	52.37
	14.69	11.76	3Ø.89	26.42	46.74	38.28
	23.12	15.26	56.33	3Ø.97	75.Ø8	47.28
Nb**	2.66	3.Ø5	17.Ø9	13.66	42.67	45.75
	1.41	Ø.95	3.28	4.1	9.29	1Ø.87
	6.99	1Ø.Ø5	33.85	42.98	54.Ø1	6Ø.82
	Ø.83	Ø.99	4.77	5.69	18.76	23.14
	16.54	19.12	34.19	34.38	5Ø.89	51.77
	28.44	21.32	54.55	33.95	72.62	49.11
	19.97	15.59	41.31	32.78	6Ø.Ø9	49.54
	16.54	24.86	37.41	48.78	55.55	65.65

KEY:
- A = 2d N.brasiliensis direct assay
- E = Experiment number
- C = Control macrophages
- C* = Control macrophages + Ø.Ø2% Antigen in vitro
- Nb = N.brasiliensis-activated macrophages
- Nb* = N.brasiliensis-activated macrophages + Ø.Ø2% Antigen in vitro
- Nb** = N.brasiliensis-activated macrophages + Ø.ØØ2% Antigen in vitro

TABLE 37

2 day N.brasiliensis infections : median % fibrinolysis values and their 95% confidence limits.

2 d Nb Direct		T1		T2		T3	
Control	M	n=16	2.83	n=16	6.58	n=16	16.21
	LL		1.72		5.11		11.84
	UL		4.0		18.0		30.32
Control + 0.02% Ag	M	n=14	2.33	n=14	6.04	n=14	12.12
	LL		1.41		4.56		10.72
	UL		3.25		7.51		17.24
Nb	M	n=16	11.33	n=16	27.85	n=16	44.92
	LL		7.24		18.16		32.11
	UL		16.89		37.72		55.8
Nb +0.02% Ag	M	n=16	11.03	n=16	30.13	n=16	45.45
	LL		6.34		18.77		31.62
	UL		15.3		39.21		52.86
Nb + 0.02% Ag	M	n=16	11.3	n=16	27.24	n=16	48.33
	LL		5.52		19.03		33.94
	UL		17.78		37.85		55.93

TABLE 38

Indirect assay data for macrophages from mice inoculated 2 days previously with N.brasiliensis or normal saline. Results expressed as milliunits of urokinase and as specific activity.

Indirect Assay	Macrophage Type				
	C	C*	Nb	Nb*	Nb**
(a)	30.5	60.1	212.05	274.94	264.86
	39.95		133.08	90.0	545.85
	206.7	22.1	129.9	158.49	
	0	16.8	102.67	120.38	89.57
	4.81			235.28	202.97
	0	2.54	205.79	248.2	235.84
	2.42	5.88	213.76	169.26	258.88
	1.98		251.98		
(b)	127.0	151.0	727.0	764.0	1089.0
	298.7	110.0	1106.23	590.2	3833.2
	766.4	116.42	1235.96	1251.9	715.96
	0				
	37.99	3.67	1296.34	1067.2	902.89
	0	9.51	910.97	1103.6	451.7
	3.53		414.1	466.36	548.29
	3.12		534.81	329.22	

KEY: E = Experiment
C = Control macrophages
C* = Control macrophages + 0.02% Ag in vitro
Nb = N.brasiliensis-activated macrophages
Nb* = N.brasiliensis-activated macrophages + 0.02% Ag in vitro
Nb** = N.brasiliensis-activated macrophages + 0.002% Ag in vitro
(a) = Total milliunits of urokinase (mU UK)
(b) = Specific activity (mU UK/mg protein)

TABLE 39

2 day N.brasiliensis infection : median values and their 95% confidence limits for indirect assay. Data expressed as milliunits of urokinase and as specific activity.

Indirect Assay		Macrophage Type				
		C	C*	Nb	Nb*	Nb**
n		8	5	7	7	6
(a)	M	16.35	13.99	172.99	183.38	235.84
	LL	Ø.99	Ø.6	116.29	105.19	144.81
	UL	105.76	28.58	232.87	261.57	326.87
(b)	M	65.16	62.97	898.18	787.62	809.43
	LL	1.56	25.62	474.46	397.79	134.83
	UL	402.2	100.32	1266.15	1177.45	1484.04

KEY: C = Control macrophages
C* = Control macrophages + Ø.Ø2% Ag in vitro
Nb = N.brasiliensis-activated macrophages
Nb* = N.brasiliensis-activated macrophages + Ø.Ø2% Ag in vitro
Nb** = N.brasiliensis-activated macrophages + Ø.ØØ2% Ag in vitro
(a) = Total milliunits of urokinase (mU UK)
(b) = Specific activity (mU UK/mg protein)
n = Number of samples

TABLE 4Ø

Significance level (Mann-Whitney U-test, 2 tailed) achieved on comparison of different experimental data groups : 2 day N.brasiliensis infection.

	Direct Assay			Indirect Assay	
Data Groups Compared	T1 2 hr	T2 4 hr	T3 6 hr	Milliunits of Urokinase	Specific Activity
1,2					
1,3	1%	1%	1%	1%	1%
1,4	1%	1%	1%	1%	1%
1,5	5%	1%	1%	1%	1%
2,3	1%	1%	Ø.1%	1%	1%
2,4	1%	1%	Ø.1%	1%	1%
2,5	5%	1%	Ø.1%	1%	1%
3,4					
3,5					
4,5					

KEY: 1 = Control macrophages
2 = Control macrophages + Ø.Ø2% Ag in vitro
3 = N.brasiliensis-activated macrophages
4 = N.brasiliensis-activated macrophages + Ø.Ø2% Ag in vitro
5 = N.brasiliensis-activated macrophages + Ø.ØØ2% Ag in vitro

TABLE 41

Fibrinolysis produced by macrophages from mice inoculated 7 days previously with *N.asteroides* or saline only : direct assay. %Fibrinolysis measured at 2 hourly intervals over 6 hours.

A	T1	2 hr	T2	4 hr	T3	6 hr
C	0.48	1.58	24.2	22.41	32.26	29.9
	2.06	1.72	7.32	4.83	12.97	9.66
	0.69	0.71	1.0	1.3	2.43	2.64
	0.08	0.36	0.2	0.53	0.66	0.4
	0.59	0.98	10.5	9.96	17.02	16.34
	0.39					
	0.76		10.42	7.71	13.53	16.85
	1.13	1.47	3.05	3.19	6.37	7.02
	1.78	1.4	6.79	3.82	11.81	7.83
	2.58	2.94	3.19	5.47	8.26	9.79
	2.24	2.31	4.07	5.47	8.61	9.27
C*	1.64	1.73	17.55	19.96	23.57	23.76
	0.84	0.46	2.59	1.02	5.48	2.73
	1.65	1.14	3.52	2.48	6.61	3.67
	0.28	0.04	0.12	0.7	0.86	0.59
	0.69	0.51	4.19	3.01	13.28	8.86
	0.66	0.59	4.46	3.56	11.52	11.48
	1.4	2.0	3.15	3.25	6.26	6.56
	0.81	2.02	2.63	3.32	4.85	6.14
	1.58	1.43	2.34	3.25	5.54	7.87
	1.28	1.22	2.3	2.75	6.45	7.19
Na	2.36	1.9	13.55	11.95	18.11	17.63
	4.77	3.62	10.61	10.76	20.31	19.36
	1.48	1.36	4.9	4.37	12.9	10.33
	11.26	7.93	23.09	21.26	37.0	31.84
	1.81	0.78	9.01	18.41	26.37	22.98
	1.35	2.09	20.48	26.83	29.8	28.72
	10.81	7.66	35.9	21.75	45.71	42.54
	9.03	9.82	27.91	23.51	50.2	44.27
	8.11	14.09	26.2	39.09	47.9	51.65
	13.31	10.77	23.61	31.1	45.03	56.91
Na*	1.84	2.45	10.23	10.99	15.65	15.56
	2.36	3.94	4.63	6.9	8.66	14.89
	3.35	3.74	10.06	9.71	20.97	22.47
	15.98	15.41	26.13	25.08	42.05	40.98
	1.02	1.47	7.65	13.21	21.57	24.9
	1.22	1.65	11.55	11.58	21.92	23.97
	13.42	12.81	25.42	23.87	51.64	39.94
	11.76	7.2	25.17	23.84	45.82	39.77
	8.56	9.76	26.68	21.19	41.14	39.36
	11.71	13.32	26.27	21.39	41.74	
Na**	3.34	3.0	18.72	10.88	22.52	15.15
	2.63	2.72	9.41	9.04	16.58	17.5
	20.14	13.01	29.16	27.14	47.53	41.24
	1.16	1.74	13.64	11.1	30.44	26.8
	10.74	11.09	19.59	17.15	36.36	35.78
	14.94	9.34	24.49	18.24	39.36	42.39
	10.69	19.55	19.42	6.97	34.53	37.62
	5.91	23.9	18.28	12.78	33.18	36.15
	2.15	2.35	19.8		38.22	15.94
	11.81	1.39	19.97		36.98	25.89
	8.75		26.11		14.38	
	1.99		23.78		26.36	
			6.66			
			13.19			

KEY: A = 7d *N.asteroides* direct assay
E = Experiment number
C = Control macrophages
C* = Control macrophages + 0.02% Antigen *in vitro*
Na = *N.asteroides*-activated macrophages
Na* = *N.asteroides*-activated macrophages + 0.02% Antigen *in vitro*
Na** = *N.asteroides*-activated macrophages + 0.002% Antigen *in vitro*

TABLE 42

7 day N.asteroides infection : median % fibrinolysis values and their 95% confidence limits.

7d N.ast infect Direct		T1		T2		T3	
Control	M	n=20	1.29	n=20	5.47	n=20	9.83
	LL		0.87		3.51		7.1
	UL		1.72		8.64		14.42
Control + 0.02% Ag	M	n=20	1.11	n=20	3.0	n=20	6.89
	LL		0.81		2.41		5.2
	UL		1.42		3.74		9.92
Na	M	n=20	6.11	n=20	20.295	n=20	32.81
	LL		3.57		15.55		24.98
	UL		8.47		24.86		40.0
Na +0.02% Ag	M	n=20	7.23	n=20	17.35	n=20	30.8
	LL		3.65		12.1		22.77
	UL		9.67		22.52		38.27
Na +0.002% Ag	M	n=22	7.27	n=22	16.81	n=22	30.94
	LL		4.96		13.83		26.07
	UL		11.25		19.88		36.15

TABLE 43

Indirect assay data for macrophages PA from mice inoculated 7 days previously with N.asteroides or normal saline. Results expressed as milliunits of urokinase and as specific activity.

Indirect Assay	Macrophage Type				
	C	C*	Na	Na*	Na**
(a)	Ø	Ø	67.62	5.12	3.96
	7.18	Ø	4.28	5.14	29.2
	Ø.Ø65	8.47	7.6	2.54	5.58
	1.423	3.Ø1	4.52	34.75	24.Ø5
	4.29	Ø.Ø9	67.62	4.85	38.Ø6
	5.93	Ø	62.26	73.33	38.23
	8.74	1.Ø2	31.25	71.41	18.13
	10.77	Ø	22.51	71.Ø5	14.51
	3.Ø2	1.26	17.57	13.Ø6	14.97
	1.25		21.96	16.53	5.76
	Ø		14.17	11.97	13.69
			18.71	14.Ø8	12.27
			12.Ø3	16.53	16.Ø7
			16.83	11.97	14.37
			5.Ø	14.Ø8	
			52.61	21.96	
			15.75	19.9	
				15.47	
(b)	Ø	Ø	4Ø.48	54.36	32.17
	54.23	Ø	3Ø.77	22.34	236.4
	Ø.39	72.54	73.38	326.25	33.91
	8.57	17.49	495.85	27.5	8Ø.27
	14.24	Ø.26	24.41	197.13	135.47
	2Ø.61	Ø	134.48	371.Ø8	1Ø4.33
	28.Ø6	3.25	13Ø.39	276.36	91.91
	59.11	Ø	131.95	23.49	64.33
	8.4	3.5	99.1	75.71	5Ø.57
	3.Ø8		59.36	35.92	16.64
	Ø		75.Ø	39.55	67.85
			48.8	48.97	38.95
			88.55	1ØØ.28	78.63
			42.26	42.69	42.81
			47.Ø5		
			65.7		

KEY: C = Control macrophages
C* = Control macrophages + Ø.Ø2% Ag in vitro
Na = N.asteroides-activated macrophages
Na* = N.asteroides-activated macrophages + Ø.Ø2% Ag in vitro
Na** = N.asteroides-activated macrophages + Ø.ØØ2% Ag in vitro
(a) = Total milliunits of urokinase (mU UK)
(b) = Specific activity (mU UK/mg protein)

TABLE 44

7 day N.asteroides infection : median values and their 95% confidence limits for indirect assay. Data expressed as milliunits of urokinase and as specific activity.

		Macrophage Type				
		C	C*	Na	Na*	Na**
(a)		n=11	n=9	n=16	n=15	n=14
	M	3.64	0.63	18.24	18.12	15.91
	LL	0.72	0	11.68	10.16	10.28
	UL	6.56	4.24	36.07	42.65	25.88
(b)		n=11	n=9	n=16	n=15	n=14
	M	14.13	1.75	76.06	70.88	66.09
	LL	4.2	0	52.08	38.35	44.76
	UL	33.76	36.27	103.93	188.32	101.66

KEY: C = Control macrophages
C* = Control macrophages + 0.02% Ag in vitro
Na = N.asteroides-activated macrophages
Na* = N.asteroides-activated macrophages + 0.02% Ag in vitro
Na** = N.asteroides-activated macrophages + 0.002% Ag in vitro
(a) = Total milliunits of urokinase (mU UK)
(b) = Specific activity (mU UK/mg cell protein)
n = number of samples

TABLE 45

Significance level (Mann-Whitney U-test, 2 tailed) achieved on comparison of difference experimental data groups : 7 day N.asteroides infection.

	Direct Assay			Indirect Assay	
Data Groups Compared	T1 2 hr	T2 4 hr	T3 6 hr	Milliunits of Urokinase	Specific Activity
1.2		1%			
1.3	0.1%	0.1%	0.1%	0.1%	0.1%
1.4	0.1%	0.1%	0.1%	0.1%	1%
1.5	0.1%	0.1%	0.1%	0.1%	0.1%
2.3	0.1%	0.1%	0.1%	0.1%	0.1%
2.4	0.1%	0.1%	0.1%	0.1%	0.1%
2.5	0.1%	0.1%	0.1%	0.1%	0.1%
3.4					
3.5					
4.5					

KEY: 1 = Control macrophages
2 = Control macrophages + 0.02% Ag in vitro
3 = N.asteroides-activated macrophages
4 = N.asteroides-activated macrophages + 0.02 Ag in vitro
5 = N.asteroides-activated macrophages + 0.002 Ag in vitro

TABLE 46

Fibrinolysis produced by macrophages from mice inoculated 7 days previously with N.brasiliensis or saline only: direct assay. % Fibrinolysis measured at 2 hourly intervals over 6 hours.

A	T1 2 hr % Fibrinolysis		T2 4 hr % Fibrinolysis		T3 6 hr % Fibrinolysis	
C	0.42	0.47	2.19	1.97	8.19	7.83
	3.57	2.09	11.73	8.29	29.78	34.28
	0	0	0.72	0.69	3.53	5.2
	1.24	1.05	3.44	4.04	6.31	7.8
	1.67	1.99	7.22	5.03	30.37	21.00
	1.91	1.37	6.52	4.32	29.67	24.13
	2.09	1.51	7.77	5.77	22.59	20.71
	1.56	1.65	9.22	5.79	23.13	26.78
C*	0.44	0.59	2.58	2.57	9.54	9.16
	3.94	1.98	18.43	9.43	45.85	31.54
	0	0.14	0	0.72	1.6	5.31
	1.37	1.24	3.64	3.17	7.43	6.69
	1.66	2.84	8.09	11.18	28.77	32.5
	1.9	2.01	9.87	11.51	35.1	32.04
	1.89	1.67	7.93	7.19	31.38	29.14
	0.76	1.63	6.48	6.21	20.32	30.16
Nb	2.33	1.89	9.11	9.06	24.22	26.06
	2.95	3.36	20.35	19.54	54.26	51.5
	0.98	1.12	3.29	2.76	13.53	9.43
	4.43	4.39	17.19	11.68	33.91	25.93
	4.29	5.01	16.44	17.43	41.86	37.04
	3.06	2.61	12.15	10.99	37.87	50.89
	4.69	4.4	23.19	15.2	43.4	42.25
	1.79	4.69	6.63	13.25	35.55	43.76
Nb*	1.88	1.71	7.33	6.1	27.14	20.2
	3.45	3.52	10.94	10.95	29.74	31.04
	0.8	0.87	3.39	3.51	11.63	12.5
	4.22	6.59	13.37	20.97	24.23	35.16
	4.85	5.02	14.88	18.34	55.77	60.69
	2.53	5.6	14.47	25.42	47.27	56.48
	4.78	6.11	15.74	23.28	48.76	58.38
	4.54	5.34	22.27	24.91	56.02	52.45
Nb**	1.4	1.88	5.99	8.18	20.96	21.08
	2.68	3.04	15.94	18.28	34.38	42.58
	0.6	0.61	2.37	2.74	10.78	10.16
	5.02	2.45	13.97	8.19	29.78	19.64
	2.4	2.91	22.97	23.02	53.5	53.59
	2.88	2.42	22.25	30.79	56.95	53.34
	7.67	3.05	24.81	25.72	60.37	52.83
	5.9	2.91	30.47	26.99	62.29	54.9

KEY: A = 7 day N.brasiliensis direct assay
C = Control macrophages
C* = Control macrophages + 0.02% Antigen in vitro
Nb = N.brasiliensis-activated macrophages
Nb* = N.brasiliensis-activated macrophages + 0.02% Antigen in vitro
Nb** = N.brasiliensis-activated macrophages + 0.002% Antigen in vitro

TABLE 47

7 day N.brasiliensis infection : median % fibrinolysis values and their 95% confidence limits.

7d N.bras infect Direct		T1		T2		T3	
Control	M	n=16	1.45	n=16	5.19	n=16	18.77
	LL		0.92		3.5		13.83
	UL		1.83		7.03		25.69
Control + 0.02% Ag	M	n=16	1.5	n=16	6.53	n=16	20.57
	LL		0.95		4.33		15.88
	UL		1.96		9.35		31.1
Nb	M	n=16	3.27	n=16	13.14	n=16	36.88
	LL		2.48		9.87		28.47
	UL		4.04		16.4		43.4
Nb + 0.02% Ag	M	n=16	3.92	n=16	14.64	n=16	39.85
	LL		2.83		10.49		29.7
	UL		5.02		19.08		51.65
Nb + 0.002% Ag	M	n=16	2.73	n=16	17.59	n=16	38.99
	LL		1.91		12.5		31.5
	UL		4.03		23.92		53.47

TABLE 48

Indirect assay data for macrophage PA from mice inoculated 7 days previously with N. brasiliensis or normal saline. Results expressed as milliunits of urokinase and as specific activity.

Indirect Assay	C	C*	Nb	Nb*	Nb**
(a)	11.13	0.275	47.23	27.4	27.55
	0	0	38.74	15.38	27.15
	2.72	3.37	56.75	70.41	64.72
	20.21	2.05	46.22	83.56	38.92
	23.7	0.67	66.32	32.99	39.93
	17.0	1.96	118.92	30.5	38.49
	0	0	99.89	38.53	27.68
	0	0	106.82	24.23	42.29
	0	0	56.38	42.13	44.81
	0	0	38.75	51.51	28.12
			39.12		24.51
			16.61		21.79
			36.78		
			32.85		
			34.4		
			25.96		
(b)	26.0	18.0	250.0	88.0	110.0
	0	0	189.62	138.28	218.42
	15.92	7.24	190.0	401.88	341.3
	32.61	2.64	327.2	598.14	59.47
	60.86	2.37	408.0	85.4	47.88
	30.18	3.85	645.7	103.68	83.22
	0	0	576.2	68.64	72.88
	0	0	887.21	102.46	176.92
	0	0	159.9	187.7	215.35
	0	0	81.2	182.6	92.1
			53.6		71.66
			48.35		72.6
			131.1		
			124.22		
			118.63		
			67.8		

KEY:

- C = Control macrophages
- C* = Control macrophages + 0.02% Antigen in vitro
- Nb = N.brasiliensis-activated macrophages
- Nb* = N.brasiliensis-activated macrophages + 0.02% Antigen in vitro
- Nb** = N.brasiliensis-activated macrophages + 0.002% Antigen in vitro
- (a) = Total milliunits of urokinase (mU UK)
- (b) = Specific activity (mU UK/mg protein)

TABLE 49

7 day N.brasiliensis infection : median values and their 95% confidence limits for indirect assay. Data expressed as milliunits of urokinase and as specific activity.

Indirect Assay		Macrophage Type				
		C	C*	Nb	Nb*	Nb**
n		10	10	16	10	12
(a)	M	6.93	1.03	47.56	37.87	33.64
	LL	0	0	36.6	26.96	27.42
	UL	15.67	5.14	72.78	58.28	43.26
(b)	M	15.09	1.93	219.91	138.28	124.51
	LL	0	0	121.43	88.0	72.1
	UL	30.43	9	388.4	350.3	200.39

KEY: C = Control macrophages
C* = Control macrophages + 0.02% Antigen in vitro
Nb = N.brasiliensis-activated macrophages
Nb* = N.brasiliensis-activated macrophages + 0.02% Antigen in vitro
Nb** = N.brasiliensis-activated macrophages + 0.002% Antigen in vitro
(a) = Total milliunits of urokinase (mU UK)
(b) = Specific activity (mU UK/mg protein)

TABLE 50

Significance level (Mann-Whitney U-test, 2 tailed) achieved on comparison of different experimental data groups : 7 day N.brasiliensis infection.

	Direct Assay			Indirect Assay	
Data Groups Compared	T1 2 hr	T2 4 hr	T3 6 hr	Milliunits of Urokinase	Specific Activity
1,2					
1,3	0.1%	0.1%	0.1%	0.1%	0.1%
1,4	0.1%	0.1%	0.1%	0.1%	0.1%
1,5	1%	0.1%	0.1%	0.1%	0.1%
2,3	0.1%	0.1%	0.1%	0.1%	0.1%
2,4	0.1%	0.1%	0.1%	0.1%	0.1%
2,5	1%	0.1%	0.1%	0.1%	0.1%
3,4					
3,5					
4,5					

KEY: 1 = Control
2 = Control + 0.02% Ag in vitro
3 = N.brasiliensis-activated macrophages
4 = N.brasiliensis-activated macrophages + 0.02% Ag in vitro
5 = N.brasiliensis-activated macrophages + 0.002% Ag in vitro
* = borderline significance

TABLE 51

Fibrinolysis produced by macrophages from mice inoculated 13 days previously with N.asteroides or saline only : direct assay. Fibrinolysis measured at 2 hourly intervals over 6 hours.

A	T1 2 hr % fibrinolysis		T2 4 hr % fibrinolysis		T3 6 hr % fibrinolysis	
	Ø.77	1.1	2.94	2.58	7.23	7.24
	1.63	2.42	5.85	7.35	10.18	12.26
	1.72	2.22	13.14	9.46	20.79	21.42
	1.36	1.6	10.76	9.94	20.12	18.16
C	6.92	5.54	20.17	21.25	40.51	43.04
	5.64	7.88	20.35	24.48	45.56	37.57
	7.85	8.09	22.97	19.68	47.34	37.31
	11.61	5.19	24.22	13.52	53.54	26.67
	2.12	1.61	6.63	4.82	18.58	14.47
	1.38	1.05	3.25	5.66	6.96	10.05
	Ø.91	Ø.85	6.91	5.65	15.06	10.49
	Ø.75	6.05	6.83	18.83	12.24	44.77
C*	7.97	6.81	22.18	15.67	54.65	40.92
	9.33	7.53	24.95	19.01	59.88	53.59
	5.35	9.66	23.27	25.45	52.17	63.28
	6.97		22.21			
	2.76	1.85	7.07	5.52	14.86	12.51
	27.12	18.62	36.33	36.12	46.02	43.94
	2.86	3.06	16.95	20.95	26.75	28.71
Na	3.64	4.58	16.9	17.73	26.73	27.29
	15.7	9.57	33.73	26.85	52.76	41.59
	15.48	9.12	32.52	25.01	55.18	42.95
	12.04	14.37	29.45	32.08	47.65	49.28
	9.97	10.48	29.61	29.15	46.97	56.57
	2.28	2.81	6.06	8.77	12.86	18.55
	11.9	21.87	31.7	34.65	47.86	45.54
Na *	Ø.69	1.18	8.83	9.38	15.89	18.21
	4.85	1.64	21.81	19.35	36.86	31.00
	13.26	15.13	27.13	23.67	42.7	40.82
	16.73	24.28	30.87	30.75	49.04	52.0
	15.5	12.61	28.51	28.55	46.88	47.79
	14.74	14.91	27.78	24.57	43.11	45.16
	2.57	2.91	6.41	6.01	16.56	13.93
	21.45	15.82	33.68	35.4	45.22	39.98
	4.09	1.37	20.03	10.27	32.04	16.85
Na**	6.86	8.03	49.13	50.09	61.28	55.65
	9.86	13.58	21.39	35.2	39.29	58.79
	8.44	15.05	18.92	32.68	34.34	52.64
	13.71	18.64	31.54	29.04	57.19	46.22
	13.07	12.79	32.8	25.63	52.58	44.98

KEY:
- A = 13d N.asteroides direct assay
- E = Experiment number
- C = Control macrophages
- C* = Control macrophages + Ø.02% Antigen in vitro
- Na = N.asteroides-activated macrophages
- Na* = N.asteroides-activated macrophages + Ø.02% Antigen in vitro
- Na** = N.asteroides-activated macrophage + Ø.ØØ2% Antigen in vitro

TABLE 52

13 day N.asteroides infection : median % fibrinolysis and their 95% confidence limits.

13 d Na direct		T1		T2		T3	
C	M	n=16	4.38	n=16	14.49	n=16	28.29
	LL		1.97		9.7		18.43
	UL		6.61		18.87		37.31
C +Ø.Ø2% antigen	M	n=15	4.43	n=15	14.22	n=15	33.82
	LL		1.61		6.77		15.41
	UL		6.89		2Ø.52		5Ø.65
Na	M	n=16	9.55	n=16	25.3	n=16	38.64
	LL		6.22		18.95		3Ø.Ø8
	UL		14.1		3Ø.84		47.65
Na +Ø.Ø2% Ag	M	n=16	1Ø.48	n=16	23.8	n=16	39.17
	LL		7.22		17.31		3Ø.33
	UL		15.Ø2		28.53		45.87
Na +Ø.ØØ2% Ag	M	n=16	1Ø.61	n=16	27.59	n=16	42.93
	LL		7.45		19.85		33.26
	UL		14.Ø6		34.1		51.Ø9

TABLE 53

Indirect assay data for macrophage PA from mice inoculated 13 days previously with N.asteroides or normal saline. Results expressed as milliunits of urokinase and as specific activity.

Indirect Assay	Macrophage Type				
	C	C*	Na	Na*	Na**
(a)	1.51	2.88	37.99	5.65	18.4
	16.13	11.04	62.47	67.3	51.07
	Ø	10.65	79.49	80.01	63.65
	Ø	Ø	87.08	21.3	67.97
	1.95	1.06	89.71	72.43	70.83
	55.25	1.72	58.17	7.28	7.37
	Ø	2.21		12.66	
	Ø.83	99.46			
		5.04			
(b)	10.35	21.52	422.63	36.31	155.9
	113.95	68.08	647.35	906.95	457.16
	Ø	67.24	447.58	460.6	253.14
	Ø	Ø	534.23	66.12	135.59
	7.73	4.48	637.5	302.9	248.32
	175.26	7.33	187.74	22.19	20.47
	Ø	8.93		39.11	
	2.44	287.29			
		12.57			

KEY: C = Control macrophages
C* = Control macrophages + Ø.Ø2% antigen in vitro
Na = N.asteroides-activated macrophages
Na* = N.asteroides-activated macrophages + Ø.Ø2% antigen in vitro
Na** = N.asteroides-activated macrophages + Ø.ØØ2% antigen in vitro
(a) = Total millunits of urokinase (mU UK)
(b) = Specific activity (mU UK/mg protein)

TABLE 54

13 day N.asteroides infection : median values and their 95% confidence limits for indirect assay. Data expressed as milliunits of urokinase and as specific activity.

Indirect Assay		Macrophage Type				
		C	C*	Na	Na*	Na**
n		8	9	6	7	6
(a)	M	1.45	5.33	68.83	39.92	43.19
	LL	Ø	1.39	58.51	6.46	30.53
	UL	28.38	50.84	79.15	76.22	55.85
(b)	M	7.06	33.62	478.43	212.92	194.37
	LL	Ø	5.91	386.74	29.15	107.25
	UL	92.81	148.11	570.12	683.71	289.49

KEY: C = Control macrophages
C* = Control macrophages + Ø.Ø2% antigen in vitro
Na = N.asteroides-activated macrophages
Na* = N.asteroides-activated macrophages + Ø.Ø2% antigen in vitro
Na** = N.asteroides-activated macrophages + Ø.ØØ2% antigen in vitro
(a) = Total milliunits of urokinase (mU UK)
(b) = Specific activity (mU UK/mg cell protein)

TABLE 55

Significance level (Mann-Whitney U-test, 2 tailed) achieved on comparison of different experimental data groups : 13 day N.asteroides infection.

	Direct Assay			Indirect Assay	
Data groups compared	T1 2 hr	T2 4 hr	T3 6 hr	Milliunits of Urokinase	Specific Activity
1,2					
1,3	1%	1%	5%	1%	1%
1,4	5%	1%		5%	5%
1,5	1%	1%	5%	1%	1%
2,3	1%	1%		5%	Ø,1%
2,4	5%	1%		5%	5%
2,5	1%	1%		5%	5%
3,4					
3,5					5%
4,5					

KEY: 1 = Control macrophages
2 = Control macrophages + Ø.Ø2% antigen
3 = N.asteroides-activated macrophages
4 = N.asteroides-activated macrophages + Ø.Ø2% antigen in vitro
5 = N.asteroides-activated macrophages + Ø.ØØ2% antigen in vitro

TABLE 56

Fibrinolysis produced by macrophages from mice inoculated 13 days previously with N.brasiliensis or saline only : direct assay. Fibrinolysis measured at 2 hourly intervals over 6 hours.

13 d Nb Direct	T1	2 hr	T2	4 hr	T3	6 hr
	3.337	1.583	21.114	15.94	36.19	31.14
	3.052	3.658	8.56	13.75	23.88	26.63
	3.787	3.28	32.43	28.57	57.07	50.9
	0.678	1.123	5.29	5.25	9.63	10.04
	3.38	3.28	10.65	7.31	19.34	12.62
C	3.27	2.06	13.23	5.96	20.91	9.89
	3.32	3.13	8.9	6.88	17.92	14.04
	3.72	3.33	8.36	7.79	15.47	13.19
	3.02	2.87	16.84	18.72	33.5	36.68
	3.24	3.28	7.48	9.13	24.43	24.03
C*	0.84	1.65	6.51	6.69	9.99	11.81
	3.09	2.87	6.67	5.85	13.85	9.69
	2.07	2.53	5.08	7.11	10.6	12.77
	3.6	4.58	6.58	10.86	12.27	20.26
	0.4	3.33	1.46	9.52	2.36	21.11
	10.42	10.23	39.64	38.65	48.54	51.27
	5.44	6.18	23.34	23.78	48.41	51.12
	15.73	14.12	43.5	40.67	54.11	50.77
	3.36	2.74	14.94	17.05	25.64	24.24
Nb	5.98	4.52	15.84	14.3	26.32	20.54
	6.24	6.58	16.18	18.1	29.57	28.89
	3.27	8.42	15.12	22.27	22.93	39.56
	5.49	7.83	13.48	19.0	18.66	37.76
	8.75	6.01	35.44	21.17	50.47	28.64
	3.07	2.93	13.59	14.44	37.49	46.01
Nb*	15.38	18.49	41.37	46.57	48.91	52.48
	3.92	3.86	13.73	13.31	27.93	24.86
	2.09	6.32	8.81	13.72	17.25	24.25
	4.89	4.25	10.01	13.41	16.11	25.07
	5.63	4.72	10.63	11.52	18.19	21.2
	4.63	3.97	10.87	11.39	20.37	19.82
	7.19	11.2	27.63	35.61	46.22	49.48
	5.53	5.6	18.05	22.25	42.75	46.22
	18.65	14.92	55.57	53.03	54.53	55.03
Nb**	2.75	2.63	11.66	10.50	20.95	20.91
	12.9	4.45	24.82	8.41	34.89	15.2
	6.58	2.96	17.98	12.06	24.62	17.18
	9.5	4.77	16.77	16.31	28.42	24.74
	3.44		7.66		14.85	

KEY:

C = Control macrophages
C* = Control macrophages + 0.02% Antigen in vitro
Nb = N.brasiliensis-activated macrophages
Nb* = N.brasiliensis-activated macrophages + 0.02% Antigen in vitro
Nb**= N.brasiliensis-activated macrophages + 0.002% Antigen in vitro

TABLE 57

13 day N.brasiliensis infection : median % fibrinolysis and their 95% confidence limits.

13 d Nb direct		T1		T2		T3	
Control	M	n=16	3.2	n=16	10.71	n=16	20.45
	LL		2.23		7.62		14.485
	UL		3.42		17.725		37.31
Control + 0.02% antigen	M	n=14	2.81	n=14	7.685	n=14	16.86
	LL		1.93		6.18		11.31
	UL		3.28		11.755		23.335
Nb	M	n=16	6.77	n=16	20.6775	n=16	37.035
	LL		5.01		16.52		29.11
	UL		9.42		29.32		44.265
Nb +0.02% Ag	M	n=16	4.94	n=16	13.6575	n=16	28.4625
	LL		3.92		11.8		21.63
	UL		9.16		26.12		36.97
Nb +0.002% Ag	M	n=15	6.98	n=16	19.5775	n=16	33.575
	LL		4.45		13.635		22.785
	UL		10.65		32.345		41.735

TABLE 58

Indirect assay data for macrophage PA from mice inoculated 13 days previously with N.brasiliensis or normal saline. Results expressed as milliunits of urokinase and as specific activity.

Indirect Assay	Macrophage Type				
	C	C*	Nb	Nb*	Nb**
(a)	15.04	10.36	47.6	26.22	32.17
	9.38	16.26	44.75	41.52	9.46
	1.13	3.21	33.67	18.75	11.46
	2.6	4.24	43.2		13.67
	2.23	0.49	14.72		
	2.36	6.21			
(b)	100.94	51.04	360.6	166.16	225.92
	112.6	126.7	365.0	284.4	64.88
	2.51	9.01	47.15	132.3	44.24
	7.03	14.35	159.51		47.15
	4.58	1.22	56.83		
	4.87	14.78			

KEY: C = Control macrophages
C* = Control macrophages + 0.02% antigen in vitro
Nb = N.brasiliensis-activated macrophages
Nb* = N.brasiliensis-activated macrophages + 0.02% antigen in vitro
Nb** = N.brasiliensis-activated macrophages + 0.002% antigen in vitro
(a) = Total milliunits of urokinase (mU UK)
(b) = Specific activity (mU UK/mg protein)

TABLE 59

13 day N.brasiliensis infections : median values and their 95% confidence limits for indirect assay. Data expressed as milliunits of urokinase and as specific activity.

Indirect Assay		Macrophage Type				
		C	C*	Nb	Nb*	Nb**
n		6	6	5	3	4
(a)	M	2.6	5.43	38.44	28.18	13.12
	LL	-Ø.18	2.28	3Ø.1	13.33	4.98
	UL	5.37	8.58	46.78	43.Ø3	21.26
(b)	M	7.Ø3	14.78	2Ø3.88	187.26	6Ø.45
	LL	-14.93	-1Ø.Ø5	123.31	88.Ø9	-4.68
	UL	28.99	39.81	284.45	286.43	125.58

KEY: C = Control macrophages
C* = Control macrophages + Ø.Ø2% antigen in vitro
Nb = N.brasiliensis-activated macrophages
Nb* = N.brasiliensis-activated macrophages + Ø.Ø2% antigen in vitro
Nb** = N.brasiliensis-activated macrophages + Ø.ØØ2% antigen in vitro
(a) = Total milliunits of urokinase (mU UK)
(b) = Specific activity (mU UK/mg cell protein)

TABLE 6Ø

Significance level (Mann-Whitney U-test, 2 tailed) achieved on comparison of different experimental data groups : 13 day N.brasiliensis infection.

	Direct Assay			Indirect Assay	
Data groups compared	T1 2 hr	T2 4 hr	T3 6 hr	Milliunits of Urokinase	Specific Activity
1,2					
1,3	Ø.1%	Ø.1%	1%	1%	5% *
1,4	Ø.1%	5%		5%	5%
1,5	1%	5%	5%		
2,3	Ø.1%	Ø.1%	Ø.1%	1%	5%
2,4	Ø.1%	Ø.1%	1%	5%	5%
2,5	Ø.1%	Ø.1%	1%		
3,4		1%			
3,5				5%	
4,5					

KEY: 1 = Control macrophages
2 = Control + Ø.Ø2% antigen in vitro
3 = N.asteroides-activated macrophages
4 = N.asteroides-activated macrophages + Ø.Ø2% antigen in-vitro
5 = N.asteroides-activated macrophages + Ø.ØØ2% antigen in vitro
* = Borderline significance

TABLE 61

Fibrinolysis produced by macrophages from mice inoculated 21 days previously with N.asteroides or saline only : direct assay. Fibrinolysis measured at 2 hourly intervals over 6 hours.

21d Na Direct	T1	2 hr	T2	4 hr	T3	6 hr
	5.86	7.54	22.57	16.55	30.0	32.16
	5.97	7.51	20.57	15.97	33.92	28.19
	8.16	5.49	19.76	15.36	34.11	34.87
	9.57	5.57	22.7	18.67	35.04	35.9
	15.28	8.56	38.29	40.14	63.38	62.93
	14.96	8.64	37.61	48.5	59.93	68.25
	14.88	30.31	35.66	40.71	65.95	71.07
	14.97	38.56	42.37	35.22	60.8	64.34
C	10.94	12.24	29.21	31.16	39.82	49.99
	8.21	13.46	25.22	34.75	42.26	45.72
	13.61	18.81	31.79	30.07	42.27	50.45
	12.54	20.94	27.36	27.88	45.27	47.76
	6.81	6.57	19.33	21.45	26.94	33.83
	7.19	6.46	20.32	18.88	30.73	32.97
C*	5.67	7.4	19.58	19.63	26.51	30.46
	7.5	8.6	14.39	20.79	28.96	35.37
	22.84	31.21	40.65	47.55	55.72	59.09
	21.6	22.39	33.45	34.89	52.72	50.07
	20.64	24.48	36.28	42.10	59.46	61.37
	18.78	12.37	31.53	24.51	56.3	50.08
	21.57	17.11	14.15	25.2	28.34	49.68
	10.95	26.24	23.79	31.16	37.29	40.4
	9.41		28.79	33.23	46.38	54.21
	10.25					
	25.28	14.67	42.45	41.89	43.35	53.64
	23.97	17.65	47.96	47.45	51.52	58.65
	25.47	35.47	35.81	52.66	46.53	55.86
	13.04	32.78	40.08	53.67	50.92	54.46
	27.15	48.26	51.68	54.6	63.95	68.26
Na	19.22	62.43	51.09	59.44	62.02	67.87
	14.14	32.66	50.11	51.19	65.1	63.65
	28.97	21.02	51.97	47.48	59.89	63.06
	11.33	8.55	14.47	14.55	25.73	24.03
	9.84	16.96	16.44	17.43	27.89	26.07
	8.74	7.05	19.64	14.23	29.4	26.21
	6.47	6.02	17.94	13.09	27.74	24.85
	27.07	23.18	40.06	34.3	47.59	45.24
	13.55	20.5	41.99	40.07	52.86	45.92
	16.93	21.18	48.6	41.52	55.61	46.9
	23.83	22.6	46.13	37.68	57.72	45.47
	20.67	41.92	48.47	44.23	62.82	57.45
	12.13	45.56	40.63	52.18	63.41	60.49
	30.75	49.72	50.04	53.31	63.28	61.22
	37.06	52.9	53.57	47.13	65.61	60.33
Na*	5.85	12.92	12.11	22.22	21.88	33.79
	8.47	10.21	12.03	22.46	21.05	33.66
	7.48	9.24	10.33	21.5	20.67	34.53
	9.89		12.59		22.04	
	22.24	20.77	37.3	30.89	50.11	44.67
	22.51	12.0	39.23	37.91	51.39	50.2
	20.16	19.29	37.66	39.77	46.6	49.46
	21.12	19.98	39.39	38.86	45.4	51.22
	51.77	47.14	48.77	52.09	57.56	64.68
Na**	35.41	44.54	43.9	51.08	57.02	60.57
	77.43	53.35	47.02	64.25	59.35	62.3
	46.09	37.88	52.01	50.33	63.13	67.14
	29.74	26.25	47.22	41.37	61.99	58.66
	9.28	10.53	20.17	20.1	31.34	31.42
	12.14	14.02	22.32	21.83	38.45	29.86
	12.74	9.74	20.84	20.33	33.25	30.95
	14.05	23.03	20.12	28.43	28.02	46.47

KEY: C = Control macrophages
C* = Control macrophages + 0.02% antigen in vitro
Na = N.asteroides-activated macrophages
Na* = N.asteroides-activated macrophages + 0.02% antigen in vitro
Na**= N.asteroides-activated macrophages + 0.002% antigen in vitro

TABLE 62

21 day N.asteroides infection : median % fibrinolysis values and their 95% confidence limits.

21d N.ast infect. direct		T1		T2		T3	
C	M	n=24	11.5875	n=24	29.235	n=24	47.845
	LL		9.515		25.29		40.295
	UL		14.75		33.75		53.485
C + Ø.Ø2% antigen in vitro	M	n=22	14.62	n=22	26.885	n=22	42.985
	LL		9.78		22.39		37.36
	UL		18.595		31.445		49.33
Na	M	n=24	19.945	n=24	35.805	n=24	46.51
	LL		14.995		31.6		40.69
	UL		26.215		47.72		57.2
Na +Ø.Ø2% antigen in vitro	M	n=23	21.5125	n=23	37.3625	n=23	47.435
	LL		15.33		29.62		40.225
	UL		29.375		44.56		55.155
Na Ø.ØØ2% Ag invitro	M	n=26	24.73	n=26	37.3	n=26	48.555
	LL		17.52		30.61		43.34
	UL		32.75		44.08		55.385

TABLE 63

Indirect assay data for macrophages PA from mice inoculated 21 days previously with N.asteroides or normal saline. Results expressed as milliunits of uorkinase and as specific activity.

Indirect Assay	Macrophage Type				
	C	C*	Na	Na*	Na**
	Ø	37.99	25.97	30.81	11.96
	Ø	25.44	48.Ø	39.85	36.12
	3	8.52	30.Ø8	17.54	31.88
	Ø	16.96	29.18	26.Ø2	21.19
(a)	6.28	5.66	57.6	27.52	28.24
	7.14		16.68		21.46
	7.12		20.59		
	8.4		18.7		
	Ø	69.14	56.85	8Ø.92	24.55
	Ø	45.44	124.37	103.95	93.17
	35.76	19.65	73.81	41.44	71.4
(b)	Ø	37.61	7Ø.85	56.52	45.81
	14.04	1Ø.75	149.13	66.15	95.14
	15.49		42.31		6Ø.95
	1Ø.61		96.87		
	21.91		52.57		

KEY: C = Control macrophages
C* = Control macrophages + Ø.Ø2% antigen in vitro
Na = N.asteroides-activated macrophages
Na* = N.asteroides-activated macrophages + Ø.Ø2% ag in vitro
Na** = N.asteroides-activated macrophage + Ø.ØØ2% ag in vitro
(a) = Total milliunits of Urokinase (mU UK)
(b) = Specific activity (mU UK/mg cell protein)

TABLE 64

21 day *N.asteroides* infection : median values and their 95% confidence limits for indirect assay. Data expressed as milliunits of urokinase and as specific activity.

Indirect Assay		Macrophage Type				
		C	C*	Na	Na*	Na**
n		8	5	8	5	6
(a)	M	3.57	16.96	28.6	27.52	24.72
	LL	Ø	8.76	18.7	21.86	19.9
	UL	7.34	25.16	43.84	33.18	29.54
(b)	M	10.16	32.55	80.1	66.15	60.95
	LL	Ø	17.75	52.57	30.3	19.9
	UL	24.9	33.18	123.0	81.99	75.03

KEY: C = Control macrophages
C* = Control macrophages + Ø.02% antigen *in vitro*
Na = *N.asteroides*-activated macrophages
Na* = *N.asteroides*-activated macrophages + Ø.02% ag *in vitro*
Na** = *N.asteroides*-activated macrophage + Ø.002% ag *in vitro*
(a) = Total milliunits of Urokinase (mU UK)
(b) = Specific activity (mU UK/mg cell protein)

TABLE 65

Significance level (Mann-Whitney U-test, 2 tailed) achieved on comparison of different experimental data groups : 21 day *N.asteroides* infection.

	Direct Assay			Indirect Assay	
Data groups compared	T1 2 hr	T2 4 hr	T3 6 hr	Milliunits of Urokinase	Specific Activity
1,2				5%	
1,3	5%			Ø.1%	1%
1,4	1%	5%		Ø.1%	1%
1,5	Ø.1%	5%		Ø.1%	1%
2,3		5%			5%
2,4		5%			
2,5	1%	1%			
3,4					
3,5					
4,5					

KEY: 1 = Control macrophages
2 = Control + Ø.02% antigen *in vitro*
3 = *N.asteroides*-activated macrophages
4 = *N.asteroides*-activated macrophages + 0.02% antigen *in vitro*
5 = N.asteroides-activated macrophages + 0.002% antigen *in vitro*
* = Borderline significance

TABLE 66

Fibrinolysis produced by macrophages from mice inoculated 21 days previously with N.brasiliensis or saline only : direct assay. Fibrinolysis measured at 2 hourly intervals over 6 hours.

21 d Nb Direct	T1	2 hr	T2	4 hr	T3	6 hr
C	0.34	0.26	2.82	2.33	5.65	7.55
	1.69	0.78	6.79	3.14	15.59	12.66
	1.09	0.88	4.85	3.71	11.16	13.73
	4.27	5.49	20.76	20.7	33.74	35.12
	5.82	5.32	26.08	25.04	35.43	34.94
	5.78	5.52	19.33	17.76	34.4	34.96
	3.92	4.86	18.96	24.63	35.12	39.92
	10.94	12.24	29.21	31.16	39.82	49.99
	8.21	13.46	25.22	34.75	42.26	45.72
	13.61	18.81	31.79	30.07	42.27	50.45
	12.54	20.94	27.36	27.88	45.27	47.76
C*	0.16	0.89	2.91	3.31	6.07	6.39
	0.45	1.25	2.76	7.4	6.89	17.63
	4.29	6.47	18.75	24.62	30.04	34.13
	3.02	6.79	15.35	23.44	26.24	33.83
	6.16	6.28	17.28	24.49	27.53	35.12
	5.12	5.6	21.02	22.1	35.41	36.55
	13.09	14.21	28.3	29.64	45.55	48.54
	13.16	21.15	25.88	32.13	42.05	45.85
	14.0		30.84	34.36	49.2	51.98
	13.5					
Nb	4.86	2.1	21.15	8.69	31.03	17.47
	1.98	2.08	9.82	11.53	13.42	33.73
	2.8	1.99	10.66	12.39	23.88	22.66
	8.41	7.71	30.53	34.73	50.19	54.25
	8.99	9.32	29.99	33.5	49.19	51.93
	7.18	7.67	26.21	28.58	42.7	45.43
	9.69	9.32	34.73	33.5	42.24	41.73
	7.67		28.58		45.43	
	19.57	15.84	34.99	29.16	51.97	42.59
	20.99	18.66	33.76	32.26	50.77	43.51
	16.69	18.85	32.45	31.79	44.72	49.36
	16.28	21.04	32.74	33.59	43.7	49.57
Nb*	4.13	4.93	13.24	24.81	20.84	39.28
	1.91	2.64	8.08	7.2	27.08	28.92
	1.55	2.2	10.17	15.09	19.46	28.37
	8.98	7.36	30.74	35.58	46.81	49.7
	8.4	7.02	30.9	27.05	49.11	42.1
	6.72	6.73	30.58	28.9	46.15	42.77
	5.89	7.38	22.97	24.77	40.78	41.17
	17.99	17.3	27.55	29.67	47.21	45.82
	15.77	17.49	26.91	30.87	43.77	42.67
	15.66	16.87	25.95	26.82	38.94	41.66
	18.81	13.57	29.87	24.48	47.08	38.35
Nb**	5.37	3.59	11.92	9.13	19.36	15.26
	2.29	2.71	10.13	9.69	30.35	29.81
	2.08	2.0	10.41	13.88	20.92	25.1
	7.42	10.0	28.02	27.86	40.31	37.09
	7.29	6.64	24.39	26.43	38.13	37.25
	5.67	5.1	24.33	25.75	34.13	38.32
	7.92	10.17	28.33	27.74	40.05	41.15
	18.92	20.08	31.94	25.88	46.51	45.28
	17.74	20.14	33.34	29.14	46.38	48.13
	19.65		31.11	32.3	47.54	
	16.79				41.05	

KEY: C = Control macrophages
C* = Control macrophages + 0.02% antigen in vitro
Nb = N.brasiliensis-activated macrophages
Nb* = N.brasiliensis-activated macrophages + 0.02% antigen in vitro
Nb** = N.brasiliensis-activated macrophage + 0.002% antigen in vitro

TABLE 67

21 day N.brasiliensis infection : median % fibrinolysis and their 95% confidence limits.

21 d Nb direct		T1		T2		T3	
C	M	n=22	6.535	n=22	19.86	n=22	34.67
	LL		3.505		14.61		24.995
	UL		9.55		25.93		40.42
C +0.02% antigen in vitro	M	n=18	6.99	n=18	20.95	n=18	33.295
	LL		3.705		14.395		24.47
	UL		10.145		26.395		40.63
Nb	M	n=23	10.36	n=23	26.8725	n=23	43.125
	LL		6.285		21.46		36.01
	UL		13.185		31.49		47.28
Nb +0.02% antigen in vitro	M	n=22	9.7	n=22	25.91	n=22	41.025
	LL		6.155		19.93		34.975
	UL		12.125		28.41		44.195
Nb + 0.002% antigen in vitro	M	n=20	9.645	n=20	23.97	n=26	37.395
	LL		5.44		18.9		31.035
	UL		12.78		28.315		41.735

TABLE 68

Indirect assay data for macrophage PA from mice inoculated 21 days previously with N.brasiliensis or normal saline. Results expressed as milliunits of urokinase and as specific activity.

Indirect Assay	Macrophage Type				
	C	C*	Nb	Nb*	Nb**
(a)	7.58	2.08	16.95	9.16	13.25
	0.02	1.42	12.56	30.73	43.22
	0	0	52.03	6.66	9.32
	0	3.98	7.58	19.02	22.1
	3	3.35	25.05	13.59	13.62
	10.7	8.01	31.91	14.77	11.16
	9.37	7.25	34.58	39.75	31.85
	0		32.15	33.69	45.02
	0		47.91		
	9.11				
	9.02				
	9.24				
(b)	46.25	11.2	254.62	56.0	235.55
	0.01	6.59	94.0	138.54	201.4
	0	0	211.76	61.95	76.89
	0	8.53	45.2	73.32	70.78
	35.76	9.61	70.95	48.66	57.88
	0	31.33	100.46	53.24	40.21
	53.89	25.56	113.82	134.84	121.05
	41.6		140.04	157.35	173.96
	0		113.82		
	0				
	16.93				
	35.35				

KEY:
- C = Control macrophages
- C* = Control macrophages + 0.02% antigen in vitro
- Nb = N.brasiliensis-activated macrophages
- Nb* = N.brasiliensis-activated macrophages + 0.02% antigen in vitro
- Nb** = N.brasiliensis-activated macrophages+ 0.002% antigen in vitro
- (a) = Total milliunits of Urokinase (mU UK)
- (b) = Specific activity (mU UK/mg cell protein)

TABLE 69

21 day N.brasiliensis infection : median values and their 95% confidence limits for indirect assay. Data expressed as milliunits of urokinase and as specific activity.

Indirect Assay		Macrophage Type				
		C	C*	Nb	Nb*	Nb**
n		13	7	9	8	8
(a)	M	4.56	3.65	28.6	20.8	22.64
	LL	0.01	0.71	16.32	10.13	11.29
	UL	8.48	7.63	41.97	33.69	38.4
(b)	M	17.88	10.8	128.48	94.73	121.71
	LL	0.005	3.3	79.51	53.24	57.88
	UL	35.56	28.45	197.33	138.54	201.4

KEY: C = Control macrophages
C* = Control macrophages + 0.02% antigen in vitro
Nb = N.brasiliensis-activated macrophages
Nb* = N.brasiliensis-activated macrophages + 0.02% antigen in vitro
Nb** = N.brasiliensis-activated macrophages+ 0.002% antigen in vitro
(a) = Total milliunits of Urokinase (mU UK)
(b) = Specific activity (mU UK/mg cell protein)

TABLE 70

Significance level (Mann-Whitney U-test, 2 tailed) achieved on comparison of different experimental data groups : 21 day N.brasiliensis infection.

	Direct Assay			Indirect Assay	
Data groups compared	T1 2 hr	T2 4 hr	T3 6 hr	Milliunits of Urokinase	Specific Activity
1,2					
1,3		5%	5%	0.1%	0.1%
1,4				1%	0.1%
1,5				0.1%	0.1%
2,3		5%	5%	0.1%	0.1%
2,4				1%	0.1%
2,5				0.1%	0.1%
3,4					
3,5			5%		
4,5					

KEY: 1 = Control macrophages
2 = Control + 0.02% antigen in vitro
3 = N.brasiliensis-activated macrophages invitro
4 = N.brasiliensis-activated macrophages + 0.02% antigen in vitro
5 = N.brasiliensis-activated macrophages + 0.002% antigen in vitro
* = Borderline significance

TABLE 71

Median % fibrinolysis values for N.asteroides and N.brasiliensis over the 21 day infection period. Data from direct assay.

Macrophage Type		Time-Point (hours)	Period of Infection (days) 2	7	13	21
	Control	T1 2Hr	25.99	1.29	4.38	11.59
		T2 4Hr	53.56	5.47	14.49	29.24
		T3 6Hr	71.34	9.83	28.29	47.85
	Na	T1 2Hr	30.09	6.11	9.55	19.95
		T2 4Hr	64.15	20.29	25.3	35.81
		T3 6	78.2	32.81	38.64	46.51
Type	Control	T1 2Hr	2.83	1.45	3.2	6.54
		T2 4Hr	6.58	5.19	10.71	19.86
		T3 6Hr	16.21	18.77	20.45	34.67
	Nb	T1 2Hr	11.33	3.27	6.77	10.36
		T2 4Hr	27.85	13.14	20.68	26.87
		T3 6Hr	44.92	36.88	37.03	43.13

TABLE 72

Direct PA assay : median % fibrinolysis values for N.asteroides and N.brasiliensis expressed as percentage of their own controls (data calculated from Table 71)

Macrophage Type		Time-Point (hours)	Period of Infection (days) 2	7	13	21
	Na	T1 2Hr	115%	473%	218%	172%
		T2 4Hr	119%	370%	174%	122%
		T3 6Hr	110%	333%	137%	97%
	Nb	T1 2Hr	400%	226%	211%	159%
		T2 4Hr	423%	253%	193%	135%
		T3 6Hr	277%	196%	183%	124%

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CHAPTER 4

LYSOZYME RELEASE FROM MOUSE PERITONEAL MACROPHAGES : INFLUENCE OF IN VIVO INFECTION WITH NOCARDIA ASTEROIDES AND NOCARDIA BRASILIENSIS

4.2.4 CELL LYSATES AND CONDITIONED MEDIUM : CUMULATIVE RELEASE OF LYSOZYME AND SPECIFIC ACTIVITY. EXPERIMENTAL SERIES B

4.2.5 ASSAY OF LYSOZYME IN THE CONDITIONED MEDIUM : RELEASED OR EXTRACELLULAR LYSOZYME

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Chapter 4

LYSOZYME RELEASE FROM MOUSE PERITONEAL MACROPHAGES : INFLUENCE OF IN VIVO INFECTION WITH NOCARDIA ASTEROIDES AND NOCARDIA BRASILIENSIS

"Fleming believed in lysozyme. He believed it was bound to have a great future; he had discovered it and he had done marvellous work on it. Other scientists would follow the path he had opened. Others would purify it, advance it. With absolute confidence he used to say, "we shall hear more about lysozyme".

Lady Amalia Fleming

4.1 INTRODUCTION

4.1.1 EARLY HISTORY

Elie Metchnikoff, a pioneer in the field of immunology, was the first to emphasize the importance of phagocytes in the host-parasite relationship (Hirsch, 1965). He established that living micro-organisms were ingested by phagocytes and that most common microbes were rapidly killed and digested after being engulfed. He realised that some digestive ferment must be present in the phagocytic cells, but was not able to characterise it or even to determine whether enzymatic attack was the process responsible for their death.

In 1922, Alexander Fleming reported the discovery of a

"remarkable bacteriolytic element found in tissues and secretions". He believed that the substance was an enzyme and called it "lysozyme" (Fleming, 1922). The paper reports the widespread distribution of lysozyme and its high concentrations in hen's egg white, saliva, tears and nasal secretions; and this was the original work that established that leukocytes contained lysozyme.

The early work on the chemistry, structure, antibacterial activity and distribution of lysozyme has been extensively reviewed by Glynn (1966), Imoto et al (1972) and Osserman and Lawlor (1966).

4.1.2 CHEMICAL CHARACTERISTICS AND BACTERIOLYTIC MECHANISM

Lysozyme (muramidase, N-acetyl muramide glycanohydrase, EC 3.2.1.17) is a cationic enzyme mol wt 14,307 which catalyses the hydrolysis of N-acetyl muramic beta-1,4 N-acetyl glucosamine linkages in the bacterial cell wall. These linkages are the specific substrate for lysozyme. Bacterial cell walls consist, in general, of linear polysaccharide chains containing repeating units of N-acetyl glucosamine and N-acetyl muramic acid residues in beta-1,4 linkage (Strominger and Ghysen, 1967). A variable number of acetyl muramic acid residues have tetrapeptide chains attached in N-acetyl muramyl-L-alanine linkages. Cross links between tetrapeptide chains result in the formation of a three-dimensional macromolecular net of peptidoglycan. This net envelopes the bacterium plasma membrane and protects the organism from osmotic lysis and gives the cell wall its rigidity.

Lysozyme breaks down this protective covering and the organism is then unprotected against osmotic stress (Gabig and Babior, 1981).

The peptidoglycan substrate is present in the cell walls of both gram-positive and gram-negative bacteria and in fungi so lysozyme should be bactericidal to a wide range of organisms. Most bacteria, however, are resistant to lysozyme, either because access to the glucosidic linkages is impaired by other cell wall constituents or because the peptidoglycan itself has changed and is no longer a suitable substrate for the enzyme (Gabig and Babior, 1981). In the case of gram-negative bacteria, the surface layers containing lipopolysaccharides, lipids and proteins obscure the substrate and make it resistant to lysis (Miller, 1969). In gram-positive organisms the cell walls are more simple and the lipoprotein polysaccharide layers are absent, but other substances may be present which protect the organism against lysozymal action. Certain minimally virulent bacteria eg Micrococcus lysodeikticus, Bacillus megatherium, and fungi such as Cryptococcus neoformans are particularly susceptible to lysozyme action (Gabig and Babior, 1981), and in these organisms the specific substrate is apparently located in a position directly accessible to the enzyme (Goldstein, 1983).

Lysozyme is one of the best characterised hydrolytic enzymes. Although not identical, lysozymes produced by different species (humans, rabbits, rats and chickens) are similar in structure, molecular weight and biologic activity (Goldstein, 1983; Carroll and Martinez, 1979). Egg-white lysozyme from hens is the most

studied enzyme and is frequently used in place of mammalian enzymes in biologic studies. (Goldstein, 1983).

4.1.3 FUNCTION OF LYSOZYME

The role of lysozyme as a bactericidal agent in mammalian sera and in resistance to infection has been the subject of controversy for years (Gordon et al, 1979; Selsted and Martinez, 1978; Gordon, 1975; Martinez and Carroll, 1980, Imoto et al, 1972 : 669). Reasons for this dispute include the fact that it is not universally present in vertebrate cells and secretions (Imoto et al, 1972 : 669); most pathogenic bacteria are insensitive to its lytic activity; the lack of uniform sensitivity of gram-positive bacteria to lysis by lysozyme, in addition investigation of the bactericidal potential of the enzyme has given rise to conflicting reports. It has been suggested that these conflicting results arose from the use of non-specific methods such as bentonite to remove lysozyme from serum and its replenishment with egg-white lysozyme which has a lower killing capacity than human serum lysozyme (Gordon et al, 1979; Imoto et al, 1972 : 669; Martinez and Carroll, 1980; Selsted and Martinez, 1978). Lysozyme has been described an an enzyme of proven bactericidal capacity (Kimura and Goldstein, 1981; Elsbach, 1973; Selsted and Martinez, 1978; Biggar and Sturgess, 1977); whereas others have stated that lysozyme appears to be incapable of killing most pathogenic organisms, except in cases where other agents sensitize naturally resistant micro- organisms to the action of the enzyme (Gabig and Babior, 1981; Goldstein,

1983; Mauel, 1983; Beaman and Beaman, 1984). It has been suggested that non-pathogenic bacteria may be avirulent because of their susceptibility to lysozyme (Fleming, 1922; Fleming, 1974; Nathan, 1983; Klebanoff and Hamon, 1975 : 509). Lysozyme is of bactericidal importance primarily in the blood where it, together with other non-specific components, kills minimally virulent micro-organisms, and in this way reduces their invasive potential in establishing tissue infection (Selsted and Martinez, 1978; Goldstein, 1983). It has also been suggested that alveolar macrophage lysozyme, both intracellularly and released into the extracellular environment, may contribute to pulmonary defence (Biggar and Sturgess, 1977).

The main biological function of lysozyme is to hydrolyse the polysaccharide portion of bacterial cell walls (Goldstein, 1983; Lassar, 1983). This degradative ability is an important factor in the intracellular and extracellular dissolution of bacteria (Elsbach, 1973; Goldstein, 1983) and some authors suggest that the function of the enzyme is to digest dead micro-organisms rather than to kill them (Mauel, 1983; Nathan et al, 1980; Klebanoff and Hamon, 1975 : 509; Beaman and Beaman, 1984). Results of the studies by Brumfitt and Glynn (1961) suggested that lysozyme may play a part in the intracellular destruction of pathogenic bacteria after phagocytosis; and Biggar and Sturgess (1977) showed electronmicroscopically that bacterial lysis by lysozyme occurs extracellularly. Thus the role of the enzyme in extracellular digestion is clear (Nathan, 1983) and it is certainly responsible for the intracellular breakdown of

sensitive microbes (Mauel, 1983; Klebanoff and Hamon, 1975 : 509).

A difference of opinion regarding a correlation between microbicidal activity of macrophages for lysozyme-resistant organisms and their lysozyme content is apparent from the literature. It has been stated that mononuclear phagocyte lysozyme content does not relate to their ability to kill phagocytosed micro- organisms (Nathan, 1983; Mauel, 1983; Klebanoff and Hamon, 1975 : 509) and that the lysozyme is probably not responsible for the enhanced antimicrobial activity of activated macrophages (Nathan et al, 1980). In contrast, Kimura and Goldstein (1981) demonstrated an absence of lysozyme in alveolar macrophages with impaired antimicrobial activity as a result of exposure to ozone; and Ridley et al, (1985) found that lower levels of lysozyme secretion were associated with the persistance of viable M.leprae segregated in macrophages.

When lysozyme acts alone its most striking activity is against non-pathogenic organisms (McClelland and van Furth, 1975). However, even in these situations lysozyme often only acts on organisms killed by complement (Nathan, 1983; Martinez and Carroll, 1980). Organisms can be rendered more susceptible to bacteriolysis by the action of other leukocytic factors or agents (Goldstein, 1983; McClelland and van Furth, 1975; Beaman and Beaman, 1984; Klebanoff and Hamon, 1975 : 509; Nathan, 1980) eg specific antibody and complement (Gordon 1978; Glynn and Milne, 1965), hydrogen peroxide and ascorbic acid (Miller, 1969),

the chelating agent versene (Repaske, 1956), leukocytic factors (Neeman, Lahav and Ginsburg, 1974) and lysozyme may function synergistically with these and other microbicidal mechanisms (Nathan, 1983). Thus lysozyme is probably of little use against most pathogenic organisms, except when other agents sensitize lysozyme-resistant organisms to enzymatic action.

Several studies have suggested a possible role of lysozyme in host defence mechanisms which extends beyond its microbicidal capabilities (Di Luzio, 1979 a and b : 447). Among the biologic properties suggested for lysozyme are a direct enhancement of phagocytosis by PMN (Klockars and Roberts, 1976) and by macrophages (Thacore and Willett, 1966). These findings led to the proposal that lysozyme may function as a self-stimulatory secretory product of the macrophage (Kokoshis et al, 1978). Other studies have suggested that lysozyme may be a mediator of the antitumour functions of macrophages (Osserman et al, 1973; Di Luzio, 1979b:448; Di Luzio, 1979a; Bordin and Young, 1976). Thus the markedly elevated serum lysozyme levels in infected animals may play a role in phagocytosis and may interfere with tumour cell surface activities (Birmingham and Jeska, 1981). The release of lysozyme from macrophages is constitutive (Gordon, Todd and Cohn, 1974) and the enzyme may therefore be an important mediator of macrophage functions (Osserman, 1975; Haneberg et al, 1984).

In addition, results of a study by Gordon et al (1979) suggested that lysozyme may function in a negative feedback system to

modulate the inflammatory response. Human lysozyme was shown to be a potent inhibitor of chemotaxis and of the production of toxic oxygen radicals by stimulated PMN; and thus lysozyme may reduce tissue injury associated with the inflammatory response without concomitant compromisation of the phagocytic capacity of PMN and host defence (Gordon et al, 1979).

4.1.4 ASSAY METHODS

Lysozyme activity is determined biochemically by measuring the rate of hydrolysis of suspensions of Micrococcus leisodeikticus either spectrophotometrically (Litwack, 1955; Parry, Chandon and Shahani, 1965; Gordon, Todd and Cohn, 1974; Selsted and Martinez, 1978; Morsky, 1983) or as zones of lysis in immunodiffusion plates (Osserman and Lawlor, 1966; Klockars, Petterson and Riska, 1976; Haneberg et al, 1984; Goldstein, 1983). The values for test samples are compared with those for standards by use of hen egg-white or other purified lysozyme preparations. Enzyme activity may be detected spectrophotometrically as low as 5 ng/ml (Selsted and Martinez, 1978) and as low as 1 ng/ml by immunodiffusion methods (Osserman and Lawlor, 1966; Goldstein, 1983). McClelland and Van Furth (1975) used immunoelectrophoresis and autoradiography in their determination of sites of synthesis of lysozyme in tissues and by cells. Histochemical demonstration of lysozyme is achieved by the immunoperoxidase method (Mason and Taylor, 1975; Kimura and Goldstein, 1981; Steele, Eremin and Brown, 1983). At the cellular level lysozyme activity in mononuclear phagocytes can be demonstrated by lysis of M lysodeikticus over the individual cells (Syren and Raeste,

1971; van der Meer, 1980 : 751).

4.1.5 DISTRIBUTION OF LYSOZYME AND CLINICAL SIGNIFICANCE

The enzyme is widely distributed in mammalian tissues, as originally described by Fleming (1922; McClelland and van Furth, 1975; Gordon, 1975 : 463). Lysozyme is present in many body fluids, especially tears and breast milk. It is also found in sputum, nasal secretions, blood serum and plasma, peritoneal fluid and numerous other body fluids (Fleming, 1922; McClelland and van Furth, 1975). Mason and Taylor (1975) studied the distribution of lysozyme in normal and pathological human tissues and the reader is referred to this text for a full description. The major cell types involved in the synthesis and release of lysozyme are the monocyte and macrophage (Gordon, Todd and Cohn, 1974; McClelland and van Furth, 1975; Osserman, 1975; Di Luzio, 1975 a and b : 447; Lasser, 1983; Goldstein, 1983). Granulocytes contain lysozyme which is present in granules (Cohn and Hirsh, 1960; Klebanoff, 1975; Briggs, Perillie and Finch, 1966; Syren and Raeste, 1971). In contrast to macrophages and monocytes, which continuously release lysozyme, neutrophils retain the enzyme within lysozomes and only release it in the course of degranulation (Osserman, 1975; Lasser, 1983) and cell death (McClelland and van Furth, 1975; Rea and Taylor, 1977). Mature neutrophils do not synthesize lysozyme but release preformed enzyme into the extracellular milieu; however these cells die after only a few hours in culture without further

production of enzyme (Gordon, Todd and Cohn, 1974). Several studies have revealed that lymphocytes do not contain lysozyme and do not synthesize it (Briggs, Perillie and Finch, 1966; Syren and Raeste, 1971; Gordon, Todd and Cohn, 1974; McClelland and van Furth, 1975).

Lysozyme has been clearly demonstrated to be elevated in certain diseases - these include diseases characterized by epitheloid cell granulomas, specifically sarcoidosis (Osserman and Lawlor, 1966; Di Luzio, 1979b; Rea and Taylor, 1978; Currie and Eccles, 1976; Goldstein, 1983), tuberculosis (Osserman and Lawlor 1966; Klockars, Petterson and Riska, 1976; Rea and Taylor, 1977), leprosy (Rea and Taylor, 1977), Crohn's disease (Falchuk, Perotto and Isselbacher, 1975; Goldstein, 1983), as well as in monocytic and myelomonocytic leukaemia (Osserman and Lawlor, 1966) and in myeloproliferative disorders (Gordon, Todd and Cohn, 1974; Di Luzio, 1979b). Serum lysozyme has been found to be elevated in pyogenic infections (Klockars, Petterson and Riska, 1976) and in rheumatoid arthritis (Pruzanski, Saito and Ogryzlo, 1970; Rea and Taylor, 1977).

4.1.6 LYSOZYME PRODUCTION AND SECRETION BY MONONUCLEAR PHAGOCYTES

Heise and Myrvik (1967) demonstrated that rabbit alveolar macrophages synthesize and secrete lysozyme into the medium when cultured *in vitro*, in the absence of phagocytosis. A major contribution to the field was made by Gordon, Todd and Cohn (1974) when they showed that mouse peritoneal macrophages and

human monocytes synthesize and secrete lysozyme in culture. The production of lysozyme by mouse unstimulated, thioglycolate stimulated macrophages and by human blood monocytes was shown by assessing cell lysates and conditioned medium (culture medium into which phagocytes had released enzyme) at daily intervals. The study showed that mononuclear phagocytes are active secretory cells with lysozyme as the major secretion product - Ø.75-1 microgram produced per $1x1Ø^6$ cells per day. This represents as much as 2.5% of total cell protein (Gordon, 198Ø : 1277; Gordon, Todd and Cohn, 1974) and approximately 25% of all extracellular protein (Gordon, 1978). Lysozyme production and secretion by mouse macrophages exceeds that of all other hydrolases, including Beta-galactosidase, Beta-glucuronidase and cathepsin D (Gordon, Todd and Cohn, 1974). Intracellular lysozyme levels remained relatively constant over the three day assay period, whereas extracellular lysozyme increased daily and represented 87-9Ø% of total lysozyme after three days. The studies of lysozyme secretion are important in that they clearly established that this enzyme is intended mostly for secretion and not for intracellular digestion (Unanue, 1976). All three cell types showed a similar pattern of lysozyme accumulation in the extracellular medium, with stimulated cells producing 2-3 times as much lysozyme as the same number of unstimulated macrophages. Specific activity of the enzyme, based on cell protein, is similar for the two types of macrophage, since the stimulated cells contain twice as much protein per cell (Gordon, 1975 : 464; Gordon, Todd and Cohn, 1974). Secretion of lysozyme by mononuclear phagocytes is characterized by substantial net

synthesis, a relatively constant intracellular concentration and continued accumulation in the medium over at least two weeks in culture (Gordon, 1980 : 1277).

The rate of lysozyme secretion by peritoneal macrophages is independent of cell stimulation, ie chemical activation (Rea and Taylor, 1977), massive phagocytosis, or exposure to lymphokines, and the enzyme is considered a constitutive secretion product of the macrophage (Gordon, 1980 : 1275, 1277; Gordon and Cohn, 1978).

Macrophages secrete the equivalent of their intra-cellular pool in 5-8 hours (Gordon, Todd and Cohn, 1974). Heise and Myrvik (1967) suggested that these cells synthesize lysozyme de novo, since the presence of inhibitors of protein and nucleic acid synthesis inhibits the production and secretion of lysozyme by macrophages. The biosynthesis and secretion of lysozyme during in vitro culture was confirmed by incubation of macrophages in radio-active amino acids and the identification of ^{14}C-labelled lysozyme (Gordon, Todd and Cohn, 1974; McClelland and van Furth, 1975). Inhibitors such as colchicine and cycloheximide inhibited lysozyme production rather than its release and diminished secretion is generally associated with reduced intracellular levels (Gordon, Todd and Cohn, 1974).

Schnyder and Baggiolini (1978 a and b) used other non-specific stimuli such as proteose peptone to elicit mouse peritoneal macrophages and found that lysozyme secretion rate remains at the

level of non-elicited controls despite major changes in other parameters; thus confirming the findings of Gordon, Todd and Cohn (1974).

The constitutive secretion of lysozyme is a cell-specific marker for the mononuclear phagocytes and PMN (Gordon, 1980 : 1278); and owing to its constancy, the rate of lysozyme secretion is a useful marker of macrophage viability (Baggiolini and Schnyder, 1982).

Gordon, Todd and Cohn (1974) demonstrated that, unlike PMN which have lysozyme in both azurophilic and specific granules (Gabig and Babior, 1981; Cohn and Hirsch, 1960; Gordon, 1980 : 1278), lysozyme is secreted from a source other than lysozomes in mononuclear phagocytes. In contrast to peritoneal macrophages, alveolar macrophages may release stored enzyme after phagocytosis (Cohn and Wiener, 1963b; Gordon, 1980 : 1278). Haneberg et al, (1984) studied lysozyme release in vitro from human monocytes and granulocytes in suspension - ie. that had not adhered to a nonphagocytosable surface. The workers found that the monocytes were capable of selective enzyme release on exposure to particulate stimuli similar to that of granulocytes.

4.1.7. MODULATION OF LYSOZYME LEVELS

Lysozyme physiology is affected in a variable way by stimuli which generally activate macrophages (Goldstein, 1983). Several studies have investigated lysozyme enzyme activity in macrophages from mycobacteria-treated animals, but the results are divergent.

Cohn and Wiener (1963a) compared intracellular lysozyme levels in normal and BCG-induced alveolar macrophages and found that BCG-induced cells contained a 3-fold higher lysozyme activity. Similar results were described by Mizunoe and Dannenberg (1965) for both alveolar macrophages and peritoneal cells. Leake and Myrvik (1968) were first to show increased secretion of lysozyme by alveolar macrophages activated by BCG *in vivo*, they also found increased lysozyme content of cell extracts, indicating increased synthesis of enzyme; in agreement with Cohn and Wiener (1963a). In contrast, Ferencik, Stefanovic and Kotulova (1982) compared lysozyme levels in peritoneal macrophages elicited with parafin oil i.p. or activated by i.p. administration of BCG vaccine, and found decreased levels of lysozyme in activated macrophages compared to elicited ones.

Serum lysozyme, reflecting increased production of lysozyme by monocytes and alveolar macrophages is raised in tuberculosis (Klockars, Pettersen and Riska, 1976; Osserman and Lawlor, 1966; Goldstein, 1983) and in experimental BCG-induced animal granuloma (Carr et al, 1980). Similarly, serum lysozyme levels are raised in untreated leprosy (Rea and Taylor, 1977). Rojas-Espinosa et al (1982) evaluated peritoneal macrophage lysozyme levels (intracellular) in a murine leprosy model and found that peritoneal cells harvested four months after infection had increased lysozyme levels when the enzyme was expressed in terms of cell number but not when expressed as specific activity, similar to the results of Gordon, Todd and Cohn (1974).

Lyberg, Closs and Prydz (1982) stimulated human monocytes in vitro with three different mycobacterial antigens - purified protein derivative (PPD) and sonicates of M.leprae and M.bovis BCG - and found that lysozyme release was enhanced in the presence of the sonicates but not in the presence of PPD. Ridley et al (1985) studied levels and distribution of lysozyme - positive cells and exudate in leprosy lesions. They found that lysozyme synthesis ceases after macrophage ingestion of M.leprae and lower levels of lysozyme secretion were associated with the persistence of viable M.leprae segregated within macrophages. Lysozyme level, as a measure of the activating effects of mycobacteria or macrophages, was found to distinguish functionally competent from functionally incompetent cells in the leprosy lesion (Ridley et al, 1985).

Intracellular lysozyme levels were measured in individual macrophages after in vitro infection with virulent or less virulent N.asteroides. Phagocytosis of N.asteroides in vitro did not modify intracellular lysozyme levels (Beaman and Black, 1985).

Lysozyme levels were measured in murine experimental Corynebacterium ovis infection and peripheral macrophage intracellular lysozyme levels were found to be increased (Hard, 1970); and serum lysozyme was elevated in experimental murine Brucella abortus infection (Birmingham and Jeska, 1981).

The work on production and secretion of lysozyme by macrophages

after exposure to various agents has revealed that some stimuli increase rates of lysozyme synthesis and these increases may or may not be accompanied by increased intracellular enzyme levels (Goldstein, 1983). However, from most of the studies cited it appears that although production of enzyme may be modulated, distribution, ie relative concentration of lysozyme between cell and medium, is rarely affected (Gee, Stevens and Hinman, 1980 a and b; Gordon, Todd and Cohn, 1974; Thyberg, Helgren and Blomgren, 1981; Maier and Ulevitch, 1981).

The effects of different agents on macrophage lysozyme production and secretion has been studied by several workers. An exception to the above statement regarding relative concentration of lysozyme was found by Hinman et al (1980), who examined the effects of cigarette smoking on lysozyme activity in human alveolar macrophages. Exposure to cigarette smoke caused a 5-fold increase in lysozyme secretion without significantly altering intracellular lysozyme concentration.

Maier and Ulevitch (1981) investigated the response of rabbit hepatic macrophages to lipopolysaccharide (LPS) and their data confirmed the finding of others (Gordon, Todd and Cohn, 1974) that 85-90% of all lysozyme activity is found in the culture fluid by 72 hours incubation. Presence of LPS in vitro yielded a 5-6-fold increase in the total amount of lysozyme activity in the LPS - stimulated macrophages as compared to controls.

Blau et al (1983) exposed human milk macrophages to Concanavalin

A or endotoxin and found that these treatments resulted in a decreased lysozyme concentration in the extracellular medium.

Macrophage lysozyme activity is not affected by exposure to many agents such as chemical agents of activation (Gordon, Todd and Cohn, 1974; Rea and Taylor, 1977). Exposure of alveolar macrophages to phorbol myristate acetate, an agent that stimulates many of the functional and metabolic changes that normally accompany phagocytosis, failed to increase lysozyme release (Biggar, 1978). Bray et al (1983) investigated the effect of parasitization by Leishmania mexicana mexicana on macrophage function in vitro and found that secretion of lysozyme in response known stimuli was not significantly altered by parasitization.

The effect of inhibitory agents on macrophage lysozyme production and secretion has been studied. Gee, Stevens and Hinman (198Ø a and b) showed that macrophages exposed in vitro to subtoxic concentrations of rifampin, a protein synthesis inhibitor, exhibited a concentration-dependent diminution in lysozyme activity but did not affect its distribution between cell and medium; impaired synthesis of lysozyme necessarily limits the secretion of the enzyme, however rifampin does not appear to affect the secretory process per se. Similarly, in cultures treated with colchicine, an antimicrotubular drug, the total amount of lysozyme was reduced due to a decrease in both intra- and extracellular enzyme levels (Thyberg, Helgren and Blomgren, 1981). The fraction present in the medium did not differ from

that in the controls, in agreement with the findings of Gordon, Todd and Cohn (1974), and although there was a partial inhibition of lysozyme production in cells lacking cytoplasmic microtubules, the mechanism of secretion is not affected.

4.1.8 LYSOZYME PRODUCTION AS A MEASURE OF MACROPHAGE FUNCTIONAL STATUS

Convincing evidence exists that lysozyme production by macrophages reflects their level of activation (Steele, Eremin and Brown, 1983). The first suggestion that elevated lysozyme levels may parallel the functional state of the reticuloendothelial system was by Cappuccino, Winston and Perri (1964; Di Luzio, 1979 a and b : 447). These workers administered BCG or E.coli bacterial endotoxin to mice and demonstrated that specific challenge to host defence with these agents increased lysozyme levels in the kidneys and spleens of mice. A relationship between splenic hyperplasia and lysozyme activity was noted (Di Luzio, 1979b : 448). Elevated levels of this enzyme are thus observed during periods of increased resistance to infection (Unanue, 1976; Glynn, 1968).

A rise in the level of serum lysozyme with the growth of a syngeneic sarcoma in rats was shown by Currie and Eccles (1976). Serum lysozyme levels correlated with the immune status of the tumour and its macrophage content (the latter conclusion being based on the paper by Gordon, Todd and Cohn, 1974, ie that lysozyme release by macrophages is unaffected by their state of activation; a concept which has been invalidated - see 4.1.9).

The finding that the surgical removal of the tumour led to a rapid decrease in serum lysozyme levels prompted the suggestion that enzyme levels could be used as a measure of macrophage-mediated host response to the tumour.

In an elegant series of studies, Kokoshis and Di Luzio showed that serum lysozyme levels are an index of macrophage functional status (Kokoshis and Di Luzio, 1979: Kokoshis et al, 1978; reviewed by Di Luzio 1979 a and b). Macrophage activation induced by glucan (Di Luzio, 1979b) was characterised by a significant enhancement in serum lysozyme concentration, hyper-phagocytosis, macrophage hyperplasia, increased resistance to S.aureus infection and hepatosplenomegaly (Kokoshis et al, 1978), in contrast to unaltered activities of several lysozomal enzymes and SGOT. Glucan therefore appears to be capable of selectively influencing lysozyme production (Di Luzio, 1979a). In addition, the elevation of serum lysozyme levels by glucan was found to be dose dependent (Kokoshis and Di Luzio, 1979). In order to determine whether increased serum lysozyme levels associated with glucan administration were due to an increased population of macrophages or to macrophage activation, methyl palmitate - an agent with potent suppressant effects on macrophages - was used to reverse the glucan stimulatory action on macrophages (Kokoshis and Di Luzio, 1979; Di Luzio, 1979b : 456). Methyl palmitate administration resulted in a decreased serum lysozyme concentration and phagocytic function. The ability of this agent to reduce lysozyme levels in glucan-treated animals with persisting hepatosplenomegaly indicates that serum lysozyme

level is an expression of macrophage functional activity rather than of macrophage pool size (Di Luzio, 1979 a and b; Kokoshis and Di Luzio, 1979).

Tilkes and Beck (1983) used lysozyme production as test for macrophage function as did Lyberg, Closs and Prydz (1982), who used lysozyme as a measure of the activating effects of mycobacteria on macrophages (Ridley et al, 1985). The same parameter was used by Steele, Eremin and Brown (1983). These workers investigated lysozyme content of monocytes and macrophages associated with human breast cancer. Significantly more monocytes from patients with cancer contained lysozyme than monocytes from comparable controls, suggesting the presence of activated circling blood monocytes. However, tumour-infiltrating macrophages were virtually devoid of the enzyme, indicating that these cells are at a low level of activation, possibly representing defective antitumour activity (Steele, Eremin and Brown, 1983).

The idea that diminished intracellular lysozyme levels and consequent diminished secretion reflects a lack of macrophage activation was also expressed by Rea and Taylor (1977), Ridley et al, (1985) and Kimura and Goldstein (1981). Rea and Taylor (1977) suggested that the lack of macrophage activation may have been a consequence of specific cell-mediated immune unresponsiveness characteristic of lepromatous leprosy. Ridley et al (1985) used lysozyme as a determinant of macrophage activation in leprosy lesions through the leprosy spectrum and

found that lysozyme distinguished functionally competent from functionally incompetent epitheloid cells, and recently recruited macrophages from those that had ingested M.leprae bacilli - lower levels of lysozyme secretion were associated with the persistence of viable organisms segregated within macrophages. After chemotherapy and in immunological upgrading reactions lysozyme secretion was increased (Ridley et al, 1985), underlining the concept that lysozyme levels are a measure of macrophage functional status.

4.1.9 THE CHOICE OF LYSOZYME AS A PARAMETER IN THIS STUDY

Lysozyme was selected as a parameter in this study primarily because production of the enzyme is an index of macrophage functional status (Cappuccino, Winston and Perri, 1964; Glynn, 1968; Rea and Taylor, 1977; Di Luzio, 1979 a and b : 460; Birmingham and Jeska, 1981; Lyberg, Closs and Prydz, 1982, Tilkes and Beck, 1983; Steele, Eremin and Brown, 1983; Ridley et al, 1985) and a characteristic of activated macrophages is increased lysozyme production (Unanue, 1976; Spicer et al, 1979; Karnovsky et al, 1975; Hopper, Wood and Nelson, 1979; Lasser, 1983).

It is well established that initial interactions between the facultative intracellular pathogen Nocardia and macrophages play a significant role in the pathogenesis of Nocardia infections (Beaman and Smathers, 1976; Beaman, 1977; Beaman, Goldstein, et al, 1978; Melendro, et al, 1978; Black et al, 1983; 1985). Nocardia infections result from unsuccessful eradication of

Nocardia organisms by phagocytes such as macrophages (Black et al, 1985). This study attempts to find a further explanation for the "apparent paradox of a micro-organism that not only is not destroyed by the phagocytic cells, but uses them to secure its existence" (Rojas-Espinosa et al, 1982). The idea that macrophage function may be modified in association with nocardial disease has been expressed frequently in this thesis. Several recent studies have correlated impairments in bactericidal capacity with reductions in intracellular lysozomal enzyme concentrations (Black et al, 1983). Goldstein et al (1978) demonstrated an absence of acid-phosphatase and Beta-glucuronidase activity in macrophages with impairments of microbicidal activity consequent to ozone exposure; and Kimura and Goldstein (1981) extended these studies by demonstrating reduced intracellular lysozyme concentrations in macrophages with defects in bactericidal activity as a result of exposure to ozone. Katoh (1981) demonstrated that macrophages infected with the intracellular parasite Mycobacterium leprae showed decreased intracellular levels of the lysozomal enzymes acid-phosphatase and Beta-glucuronidase. He suggested that this modulation of lysozomal enzyme level may contribute to the intracellular survival of the pathogen. Others have investigated the influence of M.leprae infection on intracellular lysozyme levels and on secretion (Ridley et al, 1985; section 4.1.8). Lysozyme synthesis ceases after macrophage ingestion of M.leprae and lower levels of lysozyme secretion were associated with macrophages that had ingested organisms that remained viable.

Black et al, in very recent studies (1983, 1985), revealed that the intracellular level of activity of acid-phosphatase in macrophages is reduced by *in vitro* infection with live virulent *N.asteroides* organisms, and the greater the level of intracellular infection (in terms of number of organisms) the more the level of acid-phosphatase activity was reduced (see section 1.7.4.1). The study demonstrated a relation between the virulence of the strain of *N.asteroides* and the extent of the reduction in macrophage acid-phosphatase activity (Black et al, 1983). In a related study, Black et al (1985) showed that lysozomal acid-phosphatase activity correlated with macrophage killing efficiency and is therefore an effective marker of macrophage ability to inhibit growth of or kill *N.asteroides*. Thus it appears that one of the mechanisms enabling virulent strains of *N.asteroides* to survive within macrophages is the ability of these organisms to alter lysozomal enzyme activity (Beaman et al, 1985).

Despite the differences in opinion regarding the role of lysozyme in host defence, it is an enzyme of proven bactericidal capability (Kimura and Goldstein, 1981; Biggar and Sturgess, 1977; Selsted and Martinez, 1978; Elsbach, 1973) and it appears to be accepted that lysozyme participates in the bactericidal process in cases where other agents such as antibody and complement (Martinez and Carroll, 198Ø) sensitize naturally resistant pathogenic organisms to the action of the enzyme (Gabig and Babior, 1981). In addition, a very recent study showed that neutrophils inhibit the growth of *N.asteroides* *in vitro*, in that

filament formation and amino acid uptake were inhibited for several hours. In an attempt to determine which antimicrobial components of neutrophils might contribute to this inhibition it was shown that lysozyme reduced both the amino acid uptake by, and the colony forming unit count of N.asteroides under some conditions, suggesting that lysozyme may play some part in the inhibitory process (Filice, 1985). Therefore lack of increased production and release of lysozyme in association with nocardial infection may contribute to the intracellular survival of this pathogen and lead to the development of progressive disease.

We have examined the possibility that macrophage function in N.asteroides and N.brasiliensis infections may be different. Further, macrophage function may be modulated over the 21 day infection period. It is possible that any differences in macrophage function may manifest as differences in degree of activation or suppression and it may be possible to demonstrate differences using the parameter of lysozyme production and release.

The studies by Black et al (1983, 1985) investigated the effect of in vitro infection with N.asteroides on macrophage intracellular acid-phosphatase levels. This study examines the effect of in vivo infection with both N.asteroides and N.brasiliensis on macrophage intracellular lysozyme levels and on lysozyme release.

Mononuclear phagocytes are indentified on the basis of certain

criteria and lysozyme qualifies as a cytochemical characteristic of these cells (van Furth et al, 1982) and of PMN (Gordon, Todd and Cohn 1974), however PMN do not survive overnight in culture so in the system described, lysozyme is a cell-specific marker for macrophages.

Lysozyme release is also a useful marker of cell viability (Baggiolini and Schnyder, 1982). Determination of the parameter of lysozyme production could therefore serve as a control for other parameters studied in that the presence of viable macrophages in the cultures would be confirmed.

On the basis of these considerations we have examined the parameter of lysozyme production and secretion from mouse peritoneal macrophages in N.asteroides and N.brasiliensis infections.

4.2 METHODS

The method used is adapted from that described by Parry, Chandan and Shahani (1965) and Gordon, Todd and Cohn (1974).

4.2.1 CELL CULTURES

Mouse peritoneal macrophages were harvested using standard procedures, as detailed previously, from male ICR mice (25-30g). Cells were obtained 2, 7, 13 or 21 days after intraperitoneal inoculation with live organisms of N.asteroides or N.brasiliensis, or from control, saline-inoculated mice.

Cells were pooled, concentrated by centrifugation at $4^{\circ}C$ at a RCF of 57ϐ, differential counts were performed using a Neubauer white cell counting chamber and viability was measured by trypan blue exclusion (for details of culture, see Chapter 2). Cells were resuspended in Dulbecco's medium (DB) supplemented with 2% FCS + M,P&S at a density of $2x10^6$ macrophages per ml. Peritoneal cell suspensions were plated in numbered Falcon plastic tissue culture dishes on sterile glass coverslips (22 x 22 mm) at a density of $2x10^6$ macrophages per coverslip. Cells were glass adhered for 10 minutes at $37^{\circ}C$ in the presence of 5% carbon dioxide, coverslips were then flooded with 2 ml of DB supplemented with 2% FCS + M,P&S and incubated for 2-24 hours. The cells were washed twice vigorously with HBSS to remove non-adherent cells, and replated in 1 ml of DB + 5% HIFCS + M,P&S. In each experiment as many replicate cultures of control and Nocardia-activated macrophages as possible were prepared for study of lysozyme release, the minimum number of cultures prepared was one. The number of

cultures prepared depended on the number of macrophages harvested from the control and the Nocardia-inoculated mice, and the maximum number of cultures was prepared from the cells available.

4.2.2 CUMULATIVE RELEASE OF LYSOZYME INTO CULTURE MEDIUM : EXTRACELLULAR LYSOZYME. EXPERIMENTAL SERIES "A"

"Conditioned medium" was collected from monolayer cultures at intervals of approximately 24 hours over a four day period and centrifuged at RCF of 2654 for 5 minutes at 4°C to remove cellular debris. Harvested materials were stored at -20°C until assay. Monolayer cultures wer refed with 1 ml DB + 5% HIFCS + M,P&S and reincubated.

Conditioned medium collected after 24 hours was termed sample 1 (S1) and labelled with the number and label of the culture from which it had been harvested. Similarly, conditioned medium (CM) collected after the second 24 hour incubation period was termed sample 2 (S2) and labelled with its culture number. S3 and S4 etc refer to samples collected after the third 24 hour and fourth 24 hour incubation periods.

The cumulative amounts of lysozyme released into the medium by macrophages in each culture at each sampling interval over the incubation period were determined. Cumulative enzyme concentration in the medium over the first and second day of incubation is the sum of the amount of lysozyme present in the S1 and S2 conditioned medium collected from that specific culture. The cumulative release of enzyme from the culture over the three

day period would be the sum of the enzyme concentrations in the medium at S1, S2 and S3. The cumulative release of lysozyme from macrophages in culture over a period of up to 10 days was calculated in this way.

Cell morphology was studied by phase contrast microscopy immediately prior to harvest of conditioned medium. Any cultures that appeared unhealthy were discarded (See section 2.12).

Several replicate experiments were performed at each time interval post-inoculation studied. Wherever possible, and in fact in most experiments, pooled macrophages were assessed for both plasminogen activator (PA) and lysozyme release. This was done so that both parameters could be studied in the same batch of macrophages, because lysozyme is a cell-specific marker for macrophages and release of the enzyme would establish that the cells in culture were macrophages.

After the final sample had been collected in some experiments, the cell monolayers were washed twice with warm (37°C) phosphate-buffered saline (PBS) and the cells were lysed by addition of 1000 microlitres of 0.5% Triton X-100. Tissue culture dishes were frozen and thawed once and scraped with a plastic policeman. The cell lysates were stored in Beckman microfuge tubes (Beckman Instruments, Inc., Palo Alto, California, 94304) at -20°C until Fluram assay (2.3). The specific activity of the cumulative lysozyme in the final sample was determined from these results. Experiments designated "a" in

the tables were done in this way. In some experiments determining cumulative release of lysozyme into culture medium, cell lysates were not prepared and hence specific activity was not calculated, e.g. in Table 77, specific activity was not determined for experiments 1 and 2, consequently they are not designated "a". Specific activity of cumulative lysozyme in the final sample was determined in experiments 3 and 4 and those results are shown in Table 78.

The characteristic value for lysozyme secreted by resident mouse peritoneal macrophages is given as 20-60 micrograms/milligram of protein/24 hours (Morahan, 1980).

4.2.3 EXPERIMENTAL AND CONTROL CULTURES AND EFFECT OF IN VITRO ADDITION OF NOCARDIA ANTIGEN ON MACROPHAGE LYSOZYME RELEASE

Control cultures of $2x10^6$ macropnages were always run in parallel with cultures of $2x10^6$ macrophages derived from mice previously inoculated with *N.asteroides* or *N.brasiliensis* organisms.

The effect of *in vitro* addition of culture filtrate antigens of *N.asteroides* and *N.brasiliensis* respectively on lysozyme release by cultured macrophages harvested from *N.asteroides*- or *N.brasiliensis*-inoculated mice was determined. The antigen was added to the culture medium in the desired concentration after the cells had been glass-adhered for 10 minutes. The peritoneal cells were then incubated and cumulative lysozyme release from these cultures was determined as described previously.

The concentration of homologous antigen added to cultures was Ø.ØØ2% v/v.

4.2.4 CELL LYSATES AND CONDITIONED MEDIUM : CUMULATIVE RELEASE OF LYSOZYME AND SPECIFIC ACTIVITY. EXPERIMENTAL SERIES "B".

In order to determine whether cell number in the different experimental groups viz. control, Nocardia-activated macrophages and Nocardia-activated macrophages incubated in vitro with antigen was similar at each 24 hour sampling interval, the following modification to the method described under cell cultures was made. In the initial study of lysozyme release,all the cultures were incubated over a 4 day period and conditioned medium collected at 24 hour intervals and cumulative release of lysozyme determined (Experimental series "A").

This study was designed to determine the specific activity of the lysozyme released, and confirm that trends of lysozyme release shown in the individual experiments were not due to increased or decreased cell number, or increased cell protein content. Gordon, Todd and Cohn, (1974) and Rojas-Espinosa et al (1982) both found that lysozyme activity increased in the experimental macrophage group when expressed as a function of cell number, but was similar when expressed as specific activities.

The experimental procedure was as described previously except that certain cell cultures were terminated immediately after

collection of the first sample of CM (S1) ie. after 24 hours of incubation. The CM was collected from the remainder of the cultures, which were designated for longer incubation periods, and were refed with fresh medium and returned to the incubator.

The macrophage monolayers from the cultures to be terminated were washed twice with PBS (37^{o}) and 1000 microlitres of 0.5% v/v Triton X-100 added to lyse the cells. The cell monolayer and Triton were frozen and thawed once to facilitate cell lysis (Cohn and Hirsch, 1960; Biggar and Sturgess, 1977) and the monolayers scraped using a plastic policeman. The cell lysates were collected and stored in microfuge tubes and kept at -20^{o}C until assay.

After a further 24 hours incubation (S2) certain cultures were terminated after collection of CM and cell lysates prepared. After collection of the CM from the remainder of the cultures they were refed and incubated for a further 24 hours when the S3 samples and cell lysates were collected. The samples designated to incubate for the final 24 hours (S4) were refed and returned to the incubator.

Equal numbers of cultures from each experimental group were terminated at each sampling interval whenever possible. This depended on the number of cultures in each group at the start of the experiment. In most experiments there were duplicate, triplicate or several replicate cultures for termination at each sampling interval, although in some cases there were not

sufficient cells for more than a single culture for termination. As a result of the daily termination of randomly selected cultures, there was a sequential decline in the number of dishes returned to the incubator each day, and on the fourth day the final cultures were terminated. Experiments designated "b" were done in this way.

The results obtained in this way permitted calculation of the cumulative release of lysozyme and its specific activity at each interval over the four day incubation period.

Release of enzyme after the initial 24 hour incubation period (S1) was calculated for each culture and related to the amount of cell protein present in the lysate from that culture. The amount of protein present, determined spectrofluorimetrically as described previously (2.3), gives an indication of cell number and cell protein content.

Cumulative release of enzyme after 48 hours incubation was calculated as the sum of the amount of enzyme present in the S1 and in the S2 sample for that culture. The specific activity of the lysozyme was determined by relating the total lysozyme so obtained to the amount of cell protein present in the lysate. Cumulative enzyme release and specific activity at S3 and S4 was calculated in the same way.

4.2.5 ASSAY OF LYSOZYME IN THE CONDITIONED MEDIUM : RELEASED OR EXTRACELLULAR LYSOZYME

Lysozyme present in conditioned medium was assayed by measuring the initial rate of lysis of a suspension of Micrococcus lysodeikticus (Sigma Ø128, dried cells) with the aid of a Varian Cary 219 spectrophotometer (Varian Assoc Inc, Palo Alto, California 94303) fitted with a recorder.

The lysozyme standard used was Sigma-L6876, from chicken egg-white, 3 x crystallised, Grade 1, dialysed and lyophilised. The range used for the series of standards was 1 microgram/ml, 5 micrograms/ml, 1Ø micrograms/ml, 2Ø micrograms/ml, 3Ø micrograms/ml prepared in DB + 5% HIFCS + M,P&S. Other workers have found that samples containing higher than 3Ø micrograms/ml of lysozyme deviated from linearity after 3Ø-6Ø seconds. They also demonstrated that it is necessary to use initial reaction velocities as extensive end-product inhibition is apparent above 15 micrograms of lysozyme (Parry, Chandan and Shahani, 1965).

The Micrococcus lysodeikticus dried cells were suspended in 1/15 M phosphate buffer pH 6.3 prepared as follows :

Solution A: KH_2PO_4 (Merck) 1.82 g/2Ø0 ml H_2O

Solution B: $Na_2 HPO_4.2H_2O$ (Merck) 1.19 g/1Ø0 ml H_2O

Mix 81 ml solution A with 19 ml solution B = 1/15 M phosphate buffer.

25 mg of Micrococcus lysodeikticus dried cells were suspended in 1Ø0 ml of the phosphate buffer.

Reaction conditions were as follows:

1ml of sample or standard was mixed at room temperature with 2ml Micrococcus lysodeikticus suspension, briefly stirred with an orange stick and the rate of decrease in turbidity was measured at 540 nm, using a full recording scale equal to 1 OD unit. The spectrophotometer was zeroed using distilled water and distilled water was used in the reference cuvette. The initial reaction rate was linear for at least 30 seconds and the logarithm of the reaction rate was directly proportional to the logarithm of the egg lysozyme concentration in the range 1-30 micrograms/ml. A log-log linear regression was drawn from the standards and sample concentration in microgram (µg) equivalents of chicken egg-white lysozyme were calculated from the linear regression (Fig 23).

4.2.6 ASSAY OF INTRACELLULAR LYSOZYME

Gordon (1978a) states that intracellular lysozyme levels are so low as to be barely detectable. Lysozyme present in the cell lysates was assayed spectrophotometrically by adapting the method used to assay lysozyme in conditioned medium.

Lysozyme standards were prepared in 0.5% v/v Triton X-100. The range used was 0.05 micrograms/ml, 0.1 micrograms/ml, 0.2 micrograms/ml, 0.3 micrograms/ml, 0.4 micrograms/ml, 0.5 micrograms/ml and 1 microgram/ml. Micrococcus lysodeikticus was prepared as described previously. The suspension was stirred hard using a magnetic stirrer for 30 minutes. The substrate suspension should be stable for one week at 4°C (Morsky, 1983).

The microfuge tubes of cell lysates were spun at 10 000 g for 1 minute 50 seconds in a Beckman microfuge B (Beckman Instruments Inc., Palo Alto, California 94304) to remove cellular debris.

Reaction conditions were as follows : 200 microlitres of standard or sample was mixed with 400 microlitres of cell suspension in a plastic counting tube (Greiner 115101, Germany), briefly mixed with an orange stick and poured into a 1 ml microcuvette (Spectrosil) at room temperature.

Some samples contained very low concentrations of lysozyme. To increase the sensitivity of the assay so that enzyme concentrations in these samples could be determined, 300 microlitres of sample or standard were mixed with 150 microlitres of substrate. The range of lysozyme standards prepared for assay of these samples included concentrations of 0.0125 micrograms/ml and 0.025 micrograms/ml.

The rate of decrease in turbidity was measured at 540 nm using a full recording scale equal to 0.05 OD units. The spectrophotometer was zeroed using distilled water and distilled water was used in the reference cuvette. The trace produced by low concentrations of standards and samples was often irregular for up to 3 minutes, but was linear after this period. Thereafter the reaction rate was linear for at least 2 minutes and was related to egg lysozyme concentrations by the following equation:

$Y = M [1-\exp(-ZX)]$ in the range Ø.Ø125 - 1 microgram/ml, where :

Y = rate

X = concentration

and M and Z are constants.

Sample concentrations in microgram equivalents of chicken egg-white lysozyme were calculated from the regression (Fig 24).

4.2.7 STATISTICAL TREATMENT OF DATA

To examine the significance of each experimental factor in this series of experiments, the Mann Whitney U Test (Siegel, 1956) was used to compare control and test groups. Medians and their 95% confidence limits were calculated by a method due to Tukey (Hollander and Wolfe, 1973). (For a more comprehensive discussion on statistical treatment of data, see Chapter 2.9).

4.2.8 EXPERIMENTAL SERIES "A" AND "B"

Experiments in which CM was collected at 24 hour intervals and fresh medium added to each of the cultures over 4-6 days of incubation were termed experimental series "A". In some of these experiments the specific-activity of the cumulative lysozyme in the final sample was determined, these are shown as experiment "a"; eg Table 77.

In experimental series "B", specific activity of accumulated lysozyme in culture medium was determined at each sampling interval, as detailed in 4.2.4. In series "B", intracellular and

total lysozyme levels as well as their specific activities were also determined.

The series of experiments in which the intracellular, extracellular and total lysozyme levels were determined were designated experimental series "b" and are shown as such in the tables. Experiments "a" and "b" were run concurrently and macrophages were obtained from the same cell pool.

4.3 RESULTS

4.3.1 LYSOZYME RELEASE FROM MOUSE PERITONEAL MACROPHAGES

In the initial series of experiments macrophage release of lysozyme into the culture medium was measured. In all figures and tables, data are expressed as microgram equivalents of hen's egg-white lysozyme.

Gordon, Todd and Cohn (1974) showed that there is no endogenous lysozyme present in fetal calf serum; lysozyme was stable after prolonged storage at -20°C and upon repeated freezing and thawing.

Schnyder and Baggiolini (1978a), Gordon, Todd and Cohn (1974) and Maier and Ulevitch (1981) showed that lysozyme remained fully active under culture conditions for at least 3 days.

In this system, lysozyme measured is released from macrophages, since polymorphonuclear cells die within 6-12 hours in culture (Gordon, Unkeless and Cohn, 1974; Nathan, 1983) and non-adherent cells are removed by washing prior to incubating cells in 5% HIFCS.

4.3.1.1 CALIBRATION OF THE EXPERIMENTAL SYSTEM

4.3.1.1.1 Optimum cell number

When this investigation was started, $3x10^6$ peritoneal macrophages were plated in each tissue culture dish. It was found that a large proportion of macrophages had not adhered in the initial 2-

24 hour incubation period, resulting in cultures which had widely distributed cells. This problem was overcome by reducing the cell number per dish to $2x10^6$, by introducing sterile coverslips into the tissue culture dish, and by glass adhering 1 ml of cell suspension to the coverslip at 37°C, 5% CO_2 for 10 minutes. Surface tension maintains the 1 ml cell suspension on the coverslip and after the initial adherence period the coverslip was flooded with 2 ml of DB + 2% FCS, M,P&S. Reduction of cell number reduced possible overcrowding on the coverslip and glass adhering for 10 minutes in a small volume minimized cell adherence to the plastic. This method achieved healthy cultures, as judged by morphology. It was possible to produce adequate amounts of lysozyme from $1x10^6$ macrophages cultured on glass coverslips, however there appeared to be sufficient surface area on the coverslip to maintain $2x10^6$ macrophages, so the cell density chosen was $2x10^6$ macrophages per dish (Tables 73 and 74).

4.3.1.1.2 Optimum incubation time

Measurable amounts of lysozyme were released from cultures of control and N.brasiliensis-activated macrophages after 5-8 hours of incubation. It was found that lysozyme was secreted into the same culture medium by macrophages for up to 3 days without apparent ill-effect, (Table 75, Fig 25) as judged by morphology; however maintenance of cells in the same culture medium for 4 days or more resulted in macrophages which rounded up and did not appear healthy.

Experiments were conducted in which conditioned medium was

collected at 24 hour intervals and the cultures refed with fresh medium for 8-1Ø days (Table 76). Lysozyme was released into the medium continuously over this period, and cultures appeared normal as judged by morphology. This finding is in agreement with the findings of Gordon, Todd and Cohn (1974).

It was decided that 24 hourly samples should be collected and that fresh medium should be added to the cultures at this point. Morphology was checked immediately prior to collection of the conditioned medium.

4.3.2 INFLUENCE OF TIME-COURSE OF NOCARDIA INFECTION ON LYSOZYME RELEASE FROM MOUSE PERITONEAL MACROPHAGES

Live organisms of N.asteroides or N.brasiliensis suspended in normal saline were inoculated intraperitoneally into ICR male mice. Control mice were inoculated at the same time with normal saline only. Mice were sacrificed at various intervals post-inoculation viz. 2, 7, 13 and 21 days, and the peritoneal macrophages harvested and cultured as detailed previously.

Lysozyme release from control and Nocardia-activated macrophages was measured over a four to six day period for each time interval post-inoculation and the results compared.

4.3.2.1 TWO DAY N.ASTEROIDES INFECTION

Control and N.asteroides inoculated mice were sacrificed 2 days post-inoculation and release of lysozyme into the culture medium

measured.

4.3.2.1.1 Accumulation of lysozyme in conditioned medium

Over the four day incubation period lysozyme accumulated in the medium from both Nocardia-activated and control macrophages. There was an apparent 4.9-fold increase in the amount of lysozyme which accumulated in the culture medium of the control cells over 4 days. There was a similar apparent increase (3.9-fold) in enzyme levels in the culture medium of the N.asteroides-activated macrophages over the same period. (The apparent increase in amount of lysozyme over the 4 days in culture was determined by dividing the median cumulative lysozyme value at S4 by the median lysozyme value at S1 for each of the 3 experimental groups).

Four replicate experiments were performed. The data from these experiments are presented in Table 77.

Table 78 represents the specific activity of the cumulative lysozyme released over the 4 days in culture for experiments 3a and 4a.

Median cumulative lysozyme values and their 95% confidence limits are shown in Table 109 and Fig 26 for each sampling interval (S1-S4) over the 4 days in culture. There was no statistically significant difference between the amounts of lysozyme released from control macrophages and from macrophages activated with N.asteroides two days previously (Mann Whitney U Test).

4.3.2.1.2 Effect of *in vitro* addition of culture filtrate antigens of *N.asteroides* on accumulation of macrophage lysozyme in the medium

Only one experiment was done (Experiment 4a and b) in which *N.asteroides*-activated macrophages were cultured *in vitro* with *N.asteroides* antigen for 24 hours. Apparently similar amounts of lysozyme were released by antigen-exposed activated macrophages when compared with amounts of lysozyme secreted by *N.asteroides* -activated cells, in experiment 4 (Table 77).

4.3.2.1.3 Intracellular lysozyme and percentage of lysozyme secreted (Experimental Series "B")

Two experiments (3b and 4b) were performed where the amounts of lysozyme present intracellularly were determined. These data permitted calculation of the percentage of lysozyme secreted by the cells into the culture medium (Table 79).

$$\text{Percentage lysozyme secreted} = \frac{\text{Amount of lysozyme present extracellulary}}{\text{Intracellular lysozyme} + \text{extracellular lysozyme}} \times 100$$

Gordon (1980 :1277) states that"lysozyme secretion in culture is characterized by a large net increase in total lysozyme - up to 20-fold in 3 days, 75-95% of which is in the medium". In a study of lysozyme secretion from unstimulated and thioglycolate stimulated mouse macrophages and human monocytes, Gordon, Todd and Cohn (1974) demonstrated that 87-90% of total lysozyme

production was present in the medium after 3 days. In a separate study, Gordon, Unkeless and Cohn (1974) showed that the unstimulated mouse peritoneal macrophages secreted 95% of the total lysozyme after 48 hours in culture. Macrophages stimulated by phagocytosis or agents such as endotoxin or thioglycolate medium secreted 92-98% of the total lysozyme over the same time period.

Table 79 shows that in experiments 3b and 4b control macrophages secreted 88-97% of total lysozyme after 48 hours, N.asteroides -activated macrophages secreted 78-99% and N.asteroides-activated macrophages exposed in vitro in antigen secreted 90-95% of total lysozyme over the same period of incubation. Therefore the percentage of lysozyme secreted by the macrophages did not appear to be modified by inoculation two days previously with N.asteroides, or by in vitro exposure to antigen. Similarly, percentage secretion of lysozyme did not appear to be affected after 1 and 3 days of incubation.

There was no statistically significant difference between the total lysozyme levels of control and N.asteroides-activated macrophage cultures at any of the sampling intervals (S1, S2 and S3, Mann Whitney U Test).

4.3.2.1.4 Lysozyme specific activity

The specific activity of intracellular and extracellular lysozyme was determined spectrophotometrically in two experiments (3b and 4b). The specific activity of the total amount of lysozyme was

calculated from these results. The data are presented in Table 80.

Comparison of the set of data in Table 79 with that in Table 80 shows similar trends in results when they are expressed either as micrograms of lysozyme per $2x10^6$ macrophages or when expressed as micrograms per mg of cell protein. This trend was found in Experiments 3a and 4a, where specific activity of the cumulative lysozyme at S4 was determined.

There was no statistically significant difference in the specific activities of the total lysozyme levels of control and N.asteroides-activated macrophages at any of the 3 sampling intervals (S1, S2, S3) in Experiments 3b and 4b.

4.3.2.2 TWO DAY N.BRASILIENSIS INFECTION

Control and N.brasiliensis inoculated mice were sacrificed 2 days post-inoculation, macrophages cultured and release of lysozyme determined.

4.3.2.2.1 Accumulation of lysozyme in conditioned medium

Lysozyme was released by both control and N.brasiliensis -activated macrophages and accumulated in the medium over the four - six day incubation period.

There was an apparent 4.1-fold increase in the amount of lysozyme which accumulated in the control macrophages culture medium and a similar apparent increase (3.6-fold) in the level in the N.brasiliensis-activated macrophage culture medium over the four

day incubation period.

Seven replicate experiments were performed. The data from these experiments are presented in Table 81 and the median cumulative lysozyme values and their 95% confidence limits for each sampling interval (S1-S4) in Table 11Ø and Fig 27.

Data in Table 82 represent the specific activities of cumulative enzyme in the final sample for 4 experiments. (Experiments 5 and 6 were terminated after 6 days incubation and Experiments 7a and 8a after 4 days incubation). Trends in results appear to be similar whether data are expressed in terms of cell number or in terms of specific activity.

There was no statistically significant difference between the amounts of lysozyme released from control macrophages and from macrophages activated with N.brasiliensis two days previously.

4.3.2.2.2 Effect of in vitro addition of culture filtrate antigens of N.brasiliensis on accumulation of macrophage lysozyme in the medium

The influence of incubation with antigen on the amount of lysozyme present in the N.brasiliensis-activated macrophage culture medium was determined in 3 replicate experiments (Table 81). Fig 27 and Table 11Ø show the median cumulative lysozyme values and their 95% confidence limits.

There was an apparent 2.5-fold increase in the amount of enzyme

which accumulated in the medium over 4 days.

There was no statistically significant difference between the amount of enzyme released into the culture medium by these antigen-exposed macrophages and that released by control macrophages, as determined by comparing these data using the Mann Whitney U Test.

Similarly, there was no significant difference between the amount of enzyme produced by N.brasiliensis-activated macrophages and N.brasiliensis-activated macrophages exposed in vitro to antigen. Specific activities of cumulative lysozyme in the final sample are shown in Table 82 and trends in results appear to be similar whether data are expressed as micrograms of lysozyme per $2x10^6$ macrophages or as micrograms of lysozyme per mg of cell protein.

4.3.2.2.3 Intracellular lysozyme and percentage of lysozyme secreted

Two experiments (7b and 8b) were done in which the amounts of intra- and extracellular lysozyme, total lysozyme and percentage lysozyme secreted by the macrophages were determined (Table 83).

There was no statistically significant difference in the total lysozyme levels in the 3 experimental groups over the 3 days in culture (Mann Whitney U Test).

In experiments 7b and 8b 92-94% of the total lysozyme was secreted into the medium by the control macrophages after 48 hours of incubation. Over the same period 96-97% of the total

was secreted by the N.brasiliensis-activated macrophages and 93-98% by the cells exposed in vitro to antigen. Similar percentages of enzyme were secreted by the different groups of cells after 1 and 3 days of incubation. Secretion was not affected by exposure to N.brasiliensis organisms or antigens, and all these groups of cells showed a similar pattern of lysozyme accumulation.

4.3.2.2.4 Lysozyme specific activity

Intracellular and extracellular lysozyme specific activities were determined in experiments 7b and 8b and the specific activity of the total lysozyme determined from these data (Table 84).

Comparison of the data in Tables 83 and 84 shows similar trends in the results whether they are expressed as micrograms of lysozyme per $2x10^6$ macrophages or as micrograms of lysozyme per milligram of cell protein.

There was no statistically significant difference in the total specific activites of lysozyme in the three experimental groups of cells over the 3 days in culture (Mann Whitney U Test).

4.3.2.3 SEVEN DAY N.ASTEROIDES INFECTION

Animals were sacrificed 7 days post-inoculation and release of lysozyme from cultured macrophages measured.

4.3.2.3.1 Accumulation of lysozyme in conditioned medium

There was an apparent 3.8-fold increase in lysozyme levels in the culture medium of the control macrophages and 4.3-fold increase in the level of enzyme in the medium of the N.asteroides -activated macrophages over the time of incubation.

Six replicate experiments were completed and the data from these experiments are given in Table 85. Median cumulative lysozyme values and their 95% confidence limits are shown in Table 111 and Fig 28.

Macrophages from mice inoculated 7 days previously with N.asteroides released less lysozyme into the culture medium than macrophages from mice inoculated with normal saline only. The difference was statistically significant at each 24 hour sampling interval over the four day incubation period. (S1, $p<0.01$; S2, $p<0.001$; S3, $p<0.001$; S4, $p<0.01$; Mann Whitney U Test, See Fig 28).

Table 86 gives the specific activities of cumulative lysozyme in the final sample (S4) for three experiments. Trends of results appear similar whether data are expressed in terms of cell number or specific activity.

4.3.2.3.2 Effect of in vitro addition of culture-filtrate antigens of N.asteroides on accumulation of macrophages lysozyme in the medium

Macrophages from mice inoculated 7 days previously with

N.asteroides were incubated with antigen and accumulation of enzyme in the medium measured. This was done in four replicate experiments (Table 85). Median cumulative lysozyme values and their 95% confidence limits are shown in Fig 28 and Table 111.

There was an apparent 5.7-fold increase in the amount of extracellular lysozyme in the antigen-treated cultures over the 4 day incubation period. The N.asteroides-activated macrophages incubated with antigen accumulated less lysozyme in the medium than control macrophages and this difference was statistically significant at each sampling interval. (S1, $p<0.05$; S2 $p<0.01$; S3, $p<0.01$; S4, $p<0.01$; Mann Whitney U Test).

Specific activities of cumulative lysozyme in the final sample in two experiments are shown in Table 86.

There was no statistically significant difference between the amount of lysozyme present in the CM of N.asteroides-activated macrophages and that in the CM of N.asteroides-activated macrophages incubated with antigen, as determined by the Mann Whitney U Test.

4.3.2.3.3 Intracellular lysozyme and percentage of lysozyme secreted

Table 87 shows the data from two experiments (9b and 10b) in which the amounts of intra- and extracellular lysozyme, total lysozyme and percentage released by the different experimental groups of cells were determined. In some cell lysate samples

intracellular lysozyme levels were not measurable and these levels are given as zero in the table.

80-86% of the total enzyme was secreted by the control macrophages over the 48 hour incubation period, 81-99% by the N.asteroides-activated, and 90-98% by the N.asteroides-activated cells cultured with antigen over the same period.

There was a statistically significant difference in the total lysozyme levels in the control macrophages and in the N.asteroides-activated macrophages at each sampling interval over the 3 days in culture (S1, p=0.016; S2, p=0.004; S3, p=0.004). Similarly, there was a statistically significant difference in the total lysozyme levels in control cultures and in cultures of N.asteroides-activated macrophages exposed in vitro to antigen at each sampling interval (S1, p=0.048; S2, p=0.0024; S3, p=0.01); but no statistically significant difference between N.asteroides -activated macrophages and N.asteroides-activated macrophages exposed in vitro to antigen (Mann Whitney U Test).

4.3.2.3.4 Lysozyme specific activity

The specific activity of the intra- and extracellular lysozyme and hence total lysozyme was determined in experiments 9b and 10b (Table 88).

Similar trends in results are obtained if the data are expressed in micrograms of lysozyme per $2x10^6$ macrophages or micrograms of lysozyme per mg of cell protein, however statistical significance

was not achieved at each sampling interval, as it was when data were expressed in terms of cell number. There was a significant difference between total enzyme specific activity of control and N.asteroides-activated macrophages after 48 and 72 hours of incubation (S2, p=Ø.Ø28; S3, p=Ø.ØØ6) and between control and N.asteroides-activated macrophages exposed to antigen after 72 hours of incubation (S3, p<Ø.Ø1).

4.3.2.4 SEVEN DAY N.BRASILIENSIS INFECTION

Mice were sacrificed 7 days after inoculation with N.brasiliensis, the peritoneal cells harvested and cultured, and secretion and accumulation of lysozyme by the different groups of cells measured.

4.3.2.4.1 Accumulation of lysozyme in conditioned medium

Extracellular lysozyme appeared to increase 3.4-fold in the control cultures and 3.5-fold in the N.brasiliensis-activated cell cultures over the 4 days of incubation (Table 89).

Ten replicate experiments were performed and the data from these are given in Table 89. Median cumulative lysozyme values and their 95% confidence limits are given in Table 112 and Fig 29.

Significantly less lysozyme accumulated in the CM of N.brasiliensis-activated macrophages than that of control macrophages (Mann Whitney U Test). The lysozyme levels were significantly lower in the N.brasiliensis-activated macrophage CM at each sampling interval (S1, p<Ø.ØØ1; S2, p<Ø.ØØ1; S3,

p<Ø.ØØ1; S4, p<Ø.Ø1; Mann Whitney U Test, Fig 29). Table 90 shows the specific activities of the cumulative lysozyme after the final day in culture (experiments 5, 6 and 7 were incubated over 6 days and experiment 8a over 4 days). Apparently similar trends in results occurred whether the enzyme was expressed in terms of cell number or cell protein in all three experimental cell groups.

4.3.2.4.2 Effect of *in vitro* addition of culture filtrate antigens of *N.brasiliensis* on accumulation of macrophage lysozyme in the medium

Peritoneal macrophages harvested from mice inoculated 7 days previously with *N.brasiliensis* organisms were incubated *in vitro* with *N.brasiliensis* antigen and the accumulation of lysozyme in the medium measured in five replicate experiments (Table 89). Median cumulative lysozyme values and their 95% confidence limits are shown in Table 112 and Fig 29. There was an apparent 7.3-fold increase in the amount of extracellular enzyme in the antigen-exposed cultures over the 4 days of incubation.

The antigen-exposed macrophages accumulated less lysozyme in the CM than the control macrophages. This difference was statistically significant at each sampling interval. (S1, p<Ø.Ø1; S2, p<Ø.Ø1: S3, p<Ø.Ø1; S4 p<Ø.Ø1; Mann Whitney U Test, Fig 29).

There was a significant difference between the amount of lysozyme produced by *N.brasiliensis*-activated macrophages and that

produced by those macrophages exposed in vitro to antigen after 24, 48 and 72 hours in culture (S1, $p<0.001$; S2, $p<0.05$; S3, $p=0.05$) but not after 96 hours in culture (Mann Whitney U Test). Less lysozyme was produced by the antigen-treated cultures.

4.3.2.4.3 Intracellular lysozyme and percentage of lysozyme secreted

Intra- and extracellular lysozyme levels and hence total lysozyme present was determined in one experiment (8b). From these data the percentage of lysozyme secreted by the different groups of macrophages was calculated (Table 91). Data in experiment 8b were tested for statistical difference using the Mann Whitney U Test (Siegel, 1956). In this test, statistical significance can be achieved on a minimum of $n1 = 3$ replicate samples and $n2 = 4$ replicate samples; and if there is no overlapping of the data when they are ranked, $p= 0.056$ for a two-tailed test.

There was a significant difference, within the limitations of the data, between the amount of extracellular, intracellular and total lysozyme in control cultures and those amounts in N.brasiliensis-activated macrophages after 1 day of incubation (S1, $p=0.056$ in each case). Significant differences were found between control intra- and extracellular and total lysozyme levels and those levels in cultures with antigen, after 1 day of incubation (S1, $p=0.056$ in each case).

72-86% of total enzyme was secreted by control cells, 77-94% by N.brasiliensis-activated cells and 85-89% by antigen-treated

cells after 1 day of incubation.

At the end ofthe second day of incubation, controls had secreted 76-81% of total enzyme, and N.brasiliensis-activated and antigen-treated 80-97% and 94-95% respectively.

After 3 days in culture, control macrophages had secreted 94-96%, N.brasiliensis-activated 92-97% and antigen-treated 74-86% of total lysozyme.

Although total lysozyme levels in the N.brasiliensis-activated macrophages and antigen-treated macrophages appeared to be lower than total lysozyme in controls, secretion of enzyme did not appear to be affected.

4.3.2.4.4 Lysozyme specific activity

Intracellular and extracellular specific activities of lysozyme and total enzyme specific activity are given in Table 92.

Similar trends in results are seen whether results are expressed in micrograms of lysozyme per $2x10^6$ macrophages or as micrograms of lysozyme per milligram of cell protein.

Within the limitations of the data, there is a statistically significant difference between the amount of extracellular, intracellular and total lysozyme in control cultures and the extracellular, intracellular and total lysozyme in N.brasiliensis-activated cultures ($p = 0.056$ in each case)and

between these levels in control cultures and antigen-exposed cultures (p = Ø.Ø56 in each case) after 24 hours of incubation (Mann Whitney U Test).

After 2 days of incubation there was a statistically significant difference between the amounts of extracellular enzyme in cultures of N.brasiliensis-activated macrophages and antigen-treated cultures (p = Ø.Ø56). This was found when results were expressed as micrograms of lysozyme per $2x1Ø^6$ macrophages or as micrograms of lysozyme per milligram of protein.

4.3.2.5 THIRTEEN DAY N.ASTEROIDES INFECTION

Thirteen days after inoculation with N.asteroides mice were sacrificed, the peritoneal cells harvested and cultured, and secretion and accumulation of lysozyme in the culture medium measured.

4.3.2.5.1 Accumulation of lysozyme in conditioned medium

There appeared to be a 5.4-fold increase in the amount of extracellular lysozyme in control cultures and a 4.8-fold increase in levels of enzyme in the CM of N.asteroides-activated macrophages over 4 days of incubation (Table 93).

Table 93 gives the data from four replicate experiments and Table 113 and Fig 3Ø show the median cumulative lysozyme values and their 95% confidence limits at each sampling interval.

There was no statistically significant difference between the

amount of extracellular enzyme in control cultures and that in N.asteroides-activated macrophage cultures (Mann Whitney U Test).

Table 94 shows the cumulative lysozyme specific activities of the final samples in two experiments (experiment 2 was incubated over 6 days and experiment 4a over 4 days). Trends in results are apparently similar whether expressed in terms of cell number or total protein content).

4.3.2.5.2 Effect of in vitro addition of culture filtrate antigens of N.asteroides on accumulation of macrophage lysozyme in the medium

Two experiments (3a and 4a) were performed in which the effect of in vitro exposure to N.asteroides antigens on lysozyme accumulation into the medium was determined (Table 93). Apparently similar amounts of enzyme were released by antigen-exposed cells and N.asteroides-activated cells.

4.3.2.5.3 Intracellular lysozyme and percentage of lysozyme secreted

Intra- and extracellular lysozyme levels were determined in one experiment(4b) (Table 95). The percentage of enzyme secreted by control macrophage over 3 days in culture was similar to that secreted by N.asteroides-activated macrophages, and by N.asteroides-activated macrophages exposed in vitro to antigen. The percentage of lysozyme secreted by these macrophages was similar to the percentages determined in other experiments performed at different times after inoculation of the mice, and

concurs with the data in the literature.

4.3.2.5.4 Lysozyme specific activity

Table 96 shows the intracellular, extracellular and total lysozyme specific activities.

Similar trends in results are found whether data are expressed as micrograms of lysozyme per $2x10^6$ macrophages or as micrograms of lysozyme per milligram of cell protein.

4.3.2.6 THIRTEEN DAY N.BRASILIENSIS INFECTION

Secretion and accumulation of lysozyme into the medium was measured in control macrophage cultures and in cultures of macrophages harvested from mice inoculated 13 days previously with N.brasiliensis.

4.3.2.6.1 Accumulation of lysozyme into the culture medium

Lysozyme was released into the medium by control and N.brasiliensis-activated macrophages and accumulated over 4-10 days in culture.

There was an apparent 5-fold increase in the amount of extracellular enzyme in control cultures and a 4.6-fold increase in levels of enzyme in the medium of N.brasiliensis-activated macrophages over 4 days of incubation (Table 97).

Table 97 gives the data from eight replicate experiments. The median cumulative lysozyme values and their 95% confidence limits

are presented in Table 114 and Fig 31.

There was a statistically significant difference in the amount of lysozyme present in the extracellular medium of the control and N.brasiliensis-activated macrophages after the second, third and fourth day of incubation, but not after the first day in culture (S2, $p<0.05$; S3, $p<0.05$; S4 $p<0.05$; Mann Whitney U Test, Fig 31). Less enzyme was produced by N.brasiliensis-activated macrophages.

Table 98 gives the specific activites of the cumulative lysozyme in the final sample (experiment 3 was incubated over 10 days, experiment 7 over 6 days and experiment 8a over 4 days). Trends in results are similar whether data were expressed in terms of cell number or total cell protein.

4.3.2.6.2 Effect of in vitro addition of culture filtrate antigen of N.brasiliensis on accumulation of macrophage lysozyme in the medium

Two experiments (7 and 8a) were performed in which extracellular lysozyme levels were measured in cultures of N.brasiliensis -activated macrophages after exposure to antigen (Table 97). Fig 31 and Table 114 show the median cumulative lysozyme values and their 95% confidence limits.

There was an apparent 3.2-fold increase in the amount of extracellular lysozyme over the 4 day incubation period. The pattern of accumulation of enzyme in the extracellular medium of the antigen-treated cultures appeared to be similar to that of

the N.brasiliensis-activated macrophages and there was no significant difference in the amounts of enzyme produced by these groups of cells. There was, however, a statistically significant difference between the amounts of enzyme produced by control macrophages and N.brasiliensis-activated macrophages exposed to antigen in vitro after 2, 3 and 4 days in culture(S2, $p<0.05$; S3, $p<0.05$; S4, $p<0.05$) (Mann Whitney U Test, Fig 31).

4.3.2.6.3 Intracellular lysozyme and percentage of lysozyme secreted

Intra- and extracellular lysozyme levels were measured in one experiment (8b) and total enzyme present calculated from this data (Table 99).

72-87% of total enzyme was secreted by control macrophages after 1 day of culture, 71-87% of total by N.brasiliensis-activated cells and 70-90% by antigen-treated cells over the same time period.

After 2 days in culture, controls had secreted 95% of total enzyme, N.brasiliensis-activated 93-98% and antigen-treated cells 92-98% of total enzyme. Controls secreted 95-97% of total, N.brasiliensis-activated 83-98% and antigen-treated cells 98% by the end of the third day in culture.

After 2 days of incubation, total enzyme levels in control and N.brasiliensis-activated macrophage cultures were compared, as were lysozyme levels in control and antigen-treated cultures. In

both cases total lysozyme levels were significantly different within the limitations of the data at a p value of Ø.Ø56 (Mann Whitney U Test).

4.3.2.6.4 Lysozyme specific activity

Intracellular, extracellular and total lysozyme specific activities were determined in experiment 8b (Table 1ØØ).

Total lysozyme specific activities in control and N.brasiliensis -activated cultures were significantly different after 1 day of incubation, p = Ø.Ø56.

4.3.2.7 TWENTY ONE DAY N.ASTEROIDES INFECTION

4.3.2.7.1 Accumulation of lysozyme in conditioned medium

There was an apparent 3.7-fold increase in lysozyme levels in control cultures and a 5.6-fold increase in N.asteroides -activated cells over the 4 days in culture.

Four replicate experiments were performed (Table 1Ø1). Median cumulative lysozyme values and their 95% confidence limits are shown in Table 115 and Fig 32.

Extracellular enzyme levels in N.asteroides-activated macrophage cultures were significantly less than those in control macrophage cultures at each sampling interval over the 4 days of incubation (S1, p=Ø.Ø5; S2, p<Ø.Ø1; S3, p<Ø.Ø1; S4, p<Ø.Ø1; Mann Whitney

U Test, Fig 32).

Table 102 shows the specific activities of the cumulative lysozyme in the final sample for two experiments. Macrophages were incubated over 4 days and trends in results appear similar whether data are expressed in terms of cell number or total cell protein.

4.3.2.7.2 Effect of *in vitro* addition of culture-filtrate antigens of *N.asteroides* on accumulation of macrophage lysozyme in the medium

Two experiments (3b and 4b) were done in which *N.asteroides* -activated macrophages were exposed *in vitro* to antigen and extracellular lysozyme levels measured (Table 101). Median cumulative levels are shown in Table 115 and Fig 32.

There was an apparent 5.6-fold increase in the amount of extracellular lysozyme in these cultures over the 4 day incubation period. There was a statistically significant difference between the amounts of lysozyme produced by control macropnages and by *N.asteroides*-activated macrophages exposed *in vitro* to antigen at each sampling interval over the 4 days in culture (S1, $p<0.01$; S2, $p<0.01$; S3, $p<0.01$; S4, $p<0.01$; Mann Whitney U Test, Fig 32).

The pattern of accumulation of lysozyme in the extracellular medium appeared to be similar to that of the *N.asteroides* -activated macrophages.

4.3.2.7.3 Intracellular lysozyme and percentage of lysozyme secreted

In experiments 3b and 4b intra- and extracellular enzyme levels were measured and total lysozyme calculated from these data (Table 103).

Where intracellular levels could not be measured, a value of Ø has been entered on the table.

Similar percentages of enzyme were released by the different groups of cultures at the different sampling intervals. These percentages were similar to the percentages of enzyme released by cultures of cells harvested at different times after inoculation of the animals with Nocardia.

Total lysozyme levels in control and N.asteroides-activated macrophage cultures were significantly different after 1, 2 and 3 days in culture with less lysozyme in N.asteroides-activated macrophage cultures (S1, $p = Ø.ØØ2$; S2, $p = Ø.ØØ4$; S3, $p = Ø.Ø1$). Similarly, significantly less total lysozyme was present in N.asteroides-activated macrophages exposed in vitro to antigen compared to control macrophages at each sampling interval (S1, $p = Ø.ØØ8$; S2, $p = Ø.Ø36$; S3, $p = Ø.Ø1$; Mann Whitney U Test).

4.3.2.7.4 Lysozyme specific activity

Specific activity of intracellular, extracellular and total lysozyme was determined in experiments 3b and 4b (Table 104).

Where intracellular lysozyme levels and hence specific activities could not be measured, a value of zero has been entered on the table.

Specific activities of total lysozyme in N.asteroides-activated macrophages were significantly less than those in control macrophages at each sampling interval (S1, p = Ø.ØØ2; S2, p = Ø.ØØ4; S3, p = Ø.Ø1). Similarly, total lysozyme specific activities were significantly less in antigen-treated cultures compared with controls over the 3 days of incubation (S1, p = Ø.ØØ8; S2, p = Ø.Ø36; S3, p = Ø.Ø32; Mann Whitney U Test). Results were similar whether expressed in terms of cell number or in terms of total protein content.

4.3.2.8 TWENTY ONE DAY N.BRASILIENSIS INFECTION

4.3.2.8.1 Accumulation of lysozyme in conditioned medium

Lysozyme was released by both control and N.brasiliensis -activated macrophages and accumulated in the medium over the 4-6 days in culture.

There was an apparent 5-fold increase in the levels of extracellular lysozyme in control cultures and a 6.4-fold increase in enzyme levels in N.brasiliensis-activated macrophage cultures over 4 days.

Eight replicate experiments were performed (Table 1Ø5). Median cumulative lysozyme values and their 95% confidence limits are

shown in Table 116 and Fig 33.

There was no statistically significant difference between the extracellular lysozyme levels in control cultures and those in cultures of macrophages harvested 21 days post-inoculation with N.brasiliensis. Table 106 shows the cumulative lysozyme specific activities of the final sample. (Experiments 2, 4, 6 and 7 were incubated over 6 days and experiment 8a over 4 days.) Trends in results were similar whether data were expressed in terms of cell number or total cell protein.

4.3.2.8.2 Effect of *in vitro* addition of culture-filtrate antigens of N.brasiliensis on accumulation of macrophage lysozyme in the medium

In one experiment (8a) N.brasiliensis-activated macrophages were exposed to antigen for 24 hours and extracellular lysozyme levels measured (Table 105).

The pattern of accumulation of enzyme in the extracellular medium appeared to be similar to that of the N.brasiliensis-activated cells.

4.3.2.8.3 Intracellular lysozyme and percentage of lysozyme secreted

Table 107 gives data from experiment 8b in which intra- and extracellular lysozyme levels were measured.

After 48 hours in culture, controls had secreted 82-85% of total, N.brasiliensis-activated macrophages 76-86% and antigen-treated

cells 88%. The controls had released 87-98% of total by the end of the third day in culture, the N.brasiliensis-activated 83-90% and antigen-treated 80-86%.

Similar percentages of total enzyme were secreted by control and N.brasiliensis-activated cells after the first day of incubation.

4.3.2.8.4 Lysozyme specific activity

Table 108 gives the lysozyme specific activities for experiment 8b.

Trends in results appear to be similar whether the data are expressed as micrograms of lysozyme per $2x10^6$ macrophages or as micrograms of lysozyme per milligram of cell protein.

4.3.3 RATE OF LYSOZYME ACCUMULATION IN CONDITIONED MEDIUM

Gordon, Todd and Cohn (1974) found that the rate of lysozyme production and secretion is remarkably constant for several different types of macrophages under a variety of culture conditions (section 4.1.6). He states that "the rate of lysozyme production and secretion by peritoneal macrophages is independent of cell stimulation" (Gordon, 1980 : 1277; Gordon, Todd and Cohn, 1974).

The rate of accumulation of lysozyme in conditioned medium was determined in control macrophage cultures; in cultures of macrophages derived from mice inoculated with N.asteroides and N.brasiliensis respectively; and in cultures of N.asteroides or

N.brasiliensis-activated macrophages exposed in vitro to homologous culture filtrate antigens. These rates of accumulation were determined 2, 7, 13 and 21 days post-inoculation.

Rate of accumulation of enzyme over 4 days in culture was determined by drawing linear regressions of the median cumulative lysozyme values vs time of incubation. The slopes of the linear regressions are an indication of rate of accumulation of enzyme in the medium (Figs 34-41 incl).

The general impression gained from these figures is that rate of release of enzyme from control macrophages is greater than that from Nocardia-activated macrophages. The suggestion that rate of lysozyme release from mouse peritoneal macrophages is not perturbed by stimuli was not confirmed in this study. This finding was not surprising since it is known that lysozyme physiology is affected in a variable way by stimuli prone to activate macrophages (Goldstein, 1983); in addition, decreased synthesis of enzyme (which appears to occur in Nocardia infections) necessarily is associated with diminished secretion (Gordon, Todd and Cohn, 1974).

4.3.4 DIFFERENCES IN MACROPHAGE FUNCTION IN N.ASTEROIDES AND N.BRASILIENSIS INFECTIONS

The possibility that macrophage function may be different in N.asteroides and N.brasiliensis infections was investigated by comparing lysozyme release in the two infections at 2, 7, 13 and

21 days post-inoculation. It was thought that any differences in macrophage function may manifest as differences in degree of activation or suppression. Median values for Nocardia-activated macrophage secreted lysozyme were calculated as percentage of control at each sampling interval and results compared by inspection. From Tables 109 and 110 it appears that macrophages from mice inoculated two days previously with N.asteroides secrete less lysozyme after two days in culture than macrophages from mice inoculated two days previously with N.brasiliensis (N.asteroides-activated cells secreted 63,8% of control enzyme values at S4, whereas N.brasiliensis-activated cells secreted 104.17% of control enzyme values after the same time in culture). Amounts of lysozyme secreted by N.asteroides-activated macrophages were, however, not significantly different from that secreted by controls (Mann Whitney U Test, Fig 26).

Macrophage response with respect to lysozyme release at 7 days post-inoculation appear, by inspection, to be similar in both N.asteroides and N.brasiliensis infection (Tables 111, 112).

It appears from the data (% control) shown in Tables 113, 114 that there may be a difference in macrophage function in the two infections as determined by lysozyme release by macrophages harvested 13 days post-inoculation. In addition, there was no significant difference between the amount of enzyme released by control cells and N.asteroides-activated cells, but the difference between control and N.brasiliensis-activated macrophage enzyme release was statistically significant after 2,

3 and 4 days of incubation.

Inspection of the data in Tables 115 and 116 reveals an apparent difference in 21 day N.asteroides and N.brasiliensis infections in relation to secretion of lysozyme (N.asteroides-activated macrophages secreted 41% of control lysozyme values after 4 days in culture, whereas N.brasiliensis-activated cells secreted 79% of the control enzyme value). Further, there was a significant difference between control and N.asteroides-activated cells at 21 days post-inoculation.

4.3.5 MODULATION OF LYSOZYME RELEASE OVER THE 21 DAY INFECTION PERIOD

In an attempt to determine whether lysozyme release is modulated over the 21 day period of infection in N.asteroides and N.brasiliensis infections, percentage control lysozyme values at T4 were plotted against duration of infection (Fig 42). Lysozyme release by N.asteroides-activated macrophages was similar at 2 and 13 days and at 7 and 21 days, with high levels at 2 and 13 days (apparent maximum at 13 days)and low at 7 and 21 days. In contrast, lysozyme release by N.brasiliensis-activated macrophages was maximal at 2 days, minimal at 7 days and thereafter sequentially increased at 13 and 21 days. Thus enzyme secretion appeares to be modulated in both N.asteroides and N.brasiliensis infections over the 21 day period of infection.

4.4 DISCUSSION

These studies demonstrate reduced intracellular levels and diminished secretion of lysozyme *in vitro* by *Nocardia*-activated macrophages at certain time intervals post-inoculation. In addition, the results confirm that mouse peritoneal macrophages cultured *in vitro* continuously secrete considerable amounts of lysozyme into the culture medium.

Previous workers have investigated the influence of *in vitro* infection with *N.asteroides* on intracellular lysozomal enzyme concentrations (sections 1.4.4.1; 4.1.9). The present study attempted to elucidate further the mechanisms or intracellular events enabling *Nocardia* organisms to persist and grow within macrophages.

Mononuclear phagocytes harvested from mice inoculated 2 days previously with *N.asteroides* and cultured *in vitro* produce amounts of enzyme that are not significantly different from that produced by control macrophages over the 4 day period in culture. Similarly, lysozyme production by macrophages *in vitro* is not modulated by *in vivo* inoculation with *N.brasiliensis* 2 days previously.

Macrophages harvested from mice inoculated 7 days previously with *N.asteroides* and *N.brasiliensis* respectively, produce significantly less lysozyme than cultures of control macrophages. This statistically significant difference was shown at each 24 hour sampling interval over the 4 days in culture. Therefore the

functional status of macrophages harvested from mice inoculated 7 days previously with either of these opportunitic pathogens is lowered with respect to this parameter of macrophage function. Examination of these animals prior to sacrifice revealed that the mice were ill with apparent loss of weight and harsh coat; post-mortem revealed hepatosplenomegaly and numerous abscesses in the tissues. From appearance of animals and post-mortem examination it would appear that the acute phase of infection over the period of 2-21 days post-inoculation was 2-7 days (Section 2.5.4.3.9).

There was no significant effect of inoculation 13 days previously with N.asteroides on lysozyme production by macrophages. Production of lysozyme by macrophages from mice inoculated 13 days previously with N.brasiliensis was not significantly different from that produced by control cells after 1 day in culture. However, N.brasilienis-activated macrophages produced significantly less enzyme than controls after 2, 3 and 4 days in culture. Slight modulation of production of enzyme may only manifest itself after a day in culture - a small decrease in amounts of enzyme produced per day may only become apparent when the cumulative enzyme is calculated. This decrease in amount of lysozyme produced after 2, 3 and 4 days in culture and not after 1 day may indicate that these macrophages are returning to normal functional status with respect to this parameter, as the acute phase of the infection (2 - 7 days) is passed. Prior to sacrifice animals were apparently well, only distinguishable from controls by apparent loss of weight. Post-mortem

examination again revealed marked hepatosplenomegaly and well developed, often encapsulated abscesses in the peritoneal cavity.

Similar amounts of lysozyme were produced by control macrophages and by macrophages from mice inoculated 21 days previously with N.brasiliensis, indicating a return to normal functional status with respect to this determinant of macrophage function. Post-mortem examination of these animals generally revealed markedly reduced hepatosplenomegaly and abscesses which were smaller and apparently healing or occasionally absent. These observations at post-mortem were also made for mice inoculated 21 days previously with N.asteroides. However, less lysozyme was produced by N.asteroides-activated macrophages over each 24 hour period of incubation than was produced by control cells. This difference was statistically significant after 1, 2, 3 and 4 days in culture. This finding was unexpected when related to the post-mortem examinations. It may, however, reflect the recrudescent nature of the Nocardia infection, and although the post-mortems did not reveal gross increases in nocardial activity, the apparently healing lesions may have been undergoing a repeated inflammatory process. Gonzalez-Ochoa (1973) reported on a study in which strains of Nocardia were inoculated into the foot pads of mice. One week after inoculation with N.asteroides, 90% of animals had traumatic tumoration and after 2 weeks 50% of the mice showed no sign of infection. After 3 weeks the apparently healed lesions once again showed signs of inflammation which progressed to form mycetomas after 2 months. In the same study,

using N.brasiliensis, Gonzalez-Ochoa showed an inflammation which healed and then regressed to form mycetomas over 2 months. In addition, activated macrophages may be found only at certain stages of infection (Mackaness, 1970b). It is not inconceivable that macrophages may be inhibited at various stages of infection.

Macrophages from mice previously inoculated with N.asteroides and N.brasiliensis respectively were incubated in vitro with homologous culture fitrate antigens and the influence of these antigens on lysozyme production determined.

Macrophages harvested from mice 2 days post-inoculation with N.brasiliensis and incubated with antigen produced amounts of lysozyme that were not significantly different to that produced by control cells; lysozyme production by these cells appeared to be similar to enzyme production by control cells.

Macrophages from mice harvested 7 days after inoculation with N.asteroides and exposed in vitro to antigen produced significantly less enzyme than control cells at each sampling interval over the 4 day incubation period; however, production of lysozyme by N.asteroides-activated macrophages was not modified by antigen exposure. Similarly, macrophages from mice inoculated 7 days previously with N.brasiliensis produced significantly less lysozyme than control macrophages after 1, 2, 3 and 4 days of incubation. An unexpected finding was that exposure to antigen in vitro significantly decreased lysozyme production by N.brasiliensis-activated macrophages (harvested

from mice 7 days post-inoculation) after 1, 2 and 3 days in culture. Cumulative lysozyme levels were not significantly different after 4 days of incubation. Exposure of N.asteroides-activated or N.brasiliensis-activated macrophages to antigen in vitro did not significantly modify lysozyme production at the other intervals post-inoculation studied.

Previous workers have attempted to elucidate the mechanisms of nocardial-induced alteration of macrophage function (Beaman and Black, 1985). Black et al (1983) correlated relative virulence and resistance to killing with reduction in intracellular acid-phosphatase levels. In addition, lysozomal acid-phosphatase activity correlated with macrophage killing efficiency and is therefore an effective marker of macrophage ability to inhibit growth of, or kill N.asteroides (Black et al, 1985). The same workers used the same experimental system (in vitro infection of unstimulated peritoneal macrophages with virulent and less virulent N.asteroides [Black et al, 1983]) to measure macrophage intracellular levels of lysozyme. However, intracellular lysozyme levels were not modified following phagocytosis of increasing numbers of virulent N.asteroides (Beaman and Black, 1985). The results of the present study show that macrophage intracellular lysozyme levels are decreased following infection with N.asteroides at certain time intervals post-inoculation. These conflicting results may perhaps be explained in terms of difference in experimental design - in the present study, mice were inoculated intraperitoneally with N.asteroides and the infection allowed to develop for up to 21 days, whereas in the

study by Beaman and Black (1985), washed monolayers of normal mouse macrophages were infected *in vitro* for 1 hour and then washed to remove extracellular nocardiae (Black et al, 1983). *Nocardia*-induced decrease in macrophage intracellular lysozyme levels may not occur in the absence of a full complement of immunological events; in addition, in the *in vivo* system described, statistically significant decreases in lysozyme levels were shown only after the infection had progressed for 7 days.

Percentage secretion of lysozyme appeared to be similar in the three types of macrophages studied. This is in agreement with the literature - production of lysozyme may be modulated by various agents whereas distribution between cell and medium is generally not affected (Section 4.1.7). Diminished secretion is generally associated with reduced intracellular levels since impaired synthesis necessarily limits secretion; and this phenomenon was demonstrated in the series of experiments in which lysozyme secretion was measured. The mechanism of the decrease in intracellular enzyme level associated with *Nocardia* infection was not investigated in this study, but it appears that *de novo* synthesis of the enzyme is inhibited in macrophages from mice inoculated 7 days previously with *N.asteroides* and *N.brasiliensis* and in mice inoculated 13 days previously with *N.brasiliensis* and 21 days previously with *N.asteroides*. If lysozyme was lost from within the cells it presumably would have accumulated in the medium and distribution between cell and medium would have been affected. Similarly, lysozomal degranulation is not the mechanism involved in the nocardial-

induced decrease in acid-phosphatase activity (Beaman and Black, 1985).

It is possible that inactivation of enzyme occurred, however others have shown that protein synthesis inhibitors prevent the synthesis of new lysozyme (Gordon, Todd and Cohn, 1974; Gee, Stevens and Hinman, 1980 a and b) and that macrophage protein synthesis is reduced on ingestion of M.leprae with consequent cessation of lysozyme synthesis (Ridley et al, 1985). The Nocardiae and Mycobacteriae are homogenous and closely related taxa (Lamb, Delville and Cocito, 1978); in addition Nocardia, M.tuberculosis and M.leprae are intracellular parasites (Beaman, 1976 : 19: 389; Cruickshank et al, 1973; Rojas-Espinosa et al, 1982). In macrophage cultures from infected mice the total amount of lysozyme was reduced due to a decrease in both intra- and extracellular enzyme levels, therefore the fraction present in the medium did not differ from the controls. This suggests that lysozyme production is partially inhibited, whereas the mechanism of secretion does not appear to be affected. De novo synthesis of lysozyme therefore appears to be inhibited in macrophages from mice inoculated 7 days previously with N.asteroides and N.brasiliensis and in mice inoculated 13 days previously with N.brasilienis and 21 days previously with N.asteroides.

The histopathological studies by Folb, Jaffe and Altmann (1976) suggested that aspects of macrophage function may be different in N.asteroides and N.brasiliensis infections and it was postulated

that macrophage responses may be impaired in N.brasiliensis infection. Lysozyme release was significantly reduced compared with controls at 7 and 13 days post-inoculation with N.brasiliensis. However, lysozyme release was significantly reduced compared with controls at 7 and 21 days post-inoculation in N.asteroides infection as well. Therefore comparison of macrophage lysozyme release in the two infections did not reveal any major differences in macrophage function although some differences were shown. Macrophage responses to these two Nocardia infections with respect to this parameter of macrophage function appear to be different at 13 and 21 days post-inoculation. This apparent difference is manifested by the significant differences in amounts of enzyme secreted over four days in culture by N.asteroides-activated and control macrophages at 21 days post-inoculation; and by N.brasiliensis -activated and control macrophages at 13 days post-inoculation, in addition to the trends noted on inspection of the data when expressed as percentage control enzyme value. There is also a possible difference between N.asteroides-activated cells and N.brasiliensis-activated macrophages at 2 days post-inoculation.

Macrophage function with respect to release of lysozyme appears to be modulated over the 21 day period of infection in both N.asteroides and N.brasiliensis infections. It is known that highly activated macrophages exist for only a limited period during the course of most infectious diseases (Mackaness, 1970a : 66), changing more or less gradually to reach a peak of activity and then returning to normal (Mackaness, 1970a : 67). This

finding was therefore not unexpected.

Specific activities of lysozyme within the cells and in the culture medium were determined in several representative experiments at each time interval post-inoculation studied. In the system described equal numbers of mononuclear phagocytes were distributed in tissue culture dishes at the start of the experiments. Lysozyme specific activities were measured to ensure that experimental trends were similar whether the enzyme was expressed in terms of number of macrophages cultured or in terms of total cell protein. The experiments show that lysozyme activity may be related to the amount of cellular protein without changes in trends of results as a consequence.

Decreased lysozyme intracellular levels and consequent decreased secretion of the enzyme macrophages by does not appear to be a result of toxicity since the cultures were healthy by morphological criteria (2.12), significant decreases in enzyme level did not occur at 2 days post-inoculation with either N.asteroides or N.brasiliensis, or at 13 days post-inoculation with N.asteroides or at 21 days post-inoculation with N.brasiliensis. In addition, macrophages from the same cell pool showed significant increases in plasminogen activator release (Chapter 3).

The nocardial cell walls are resistant to the action of lysozyme (Bourgeois and Beaman, 1976; Law and Marks, 1982). However, glycine potentiates the effects of lysozyme on the cell wall of

N.asteroides and N.caviae : the enzyme converted these organisms to spheroplasts which then grew as L-phase variants (Bourgeois and Beaman, 1976; see 1.7.5.3). It has been suggested that wall-deficient forms are produced by interaction with activated macrophages and that these forms are refractory to the degradative enzymes and persist in the tissues (Beaman and Scates, 1981). Whether reduction in macrophage lysozyme production and secretion is related to the development of L-forms remains to be elucidated.

The implications of decreased lysozyme production and secretion by macrophages are interesting. Several studies have correlated impairments in bactericidal capacity with reductions in intracellular lysozomal enzyme concentrations (Kimura and Goldstein, 1981; Black et al, 1985). Reductions in the intracellular levels of both the acid hydrolases (Goldstein et al, 1978; Katoh, 1981; Black et al, 1983, 1985) and in intracellular levels of lysozyme (Kimura and Goldstein, 1981; Ridley et al, 1985) have been associated with defects in bactericidal function and tumoricidal function (Steele, Eremin and Brown, 1983). Black et al (1983) suggested that reductions in acid-phosphatase activity imply organism-induced macrophage dysfunction and that these reductions suggest a general decrease in lysozomal activity in macrophages. The present study extends these observations by demonstrating reduced concentrations of lysozyme in both N.asteroides and N.brasiliensis infections at 7 days

post-inoculation; in N.brasiliensis infection at 13 days and in N.asteroides infection at 21 days post-inoculation. There is convincing evidence that lysozyme production by macrophages reflects their level of activation (Kokoshis et al, 1978; Di Luzio, 1979 a and b). The finding that production and hence secretion of lysozyme by mouse peritoneal macrophages is inhibited at certain stages of Nocardia infection suggests that at these stages the macrophages are at a low level of activity. Filice (1985) has indicated that lysozyme may play some part in host defence against N.asteroides. Reduction of intracellular levels of lysozyme may contribute to and facilitate the intracellular survival and growth of the Nocardia parasite. In addition, lysozyme may function as a self-stimulatory secretion product of the macrophage (Kokoshis et al, 1978), therefore decreased secretion of the enzyme may impair host defence mechanisms and contribute to the pathogenesis of Nocardia infections.

4.5 FIGURES

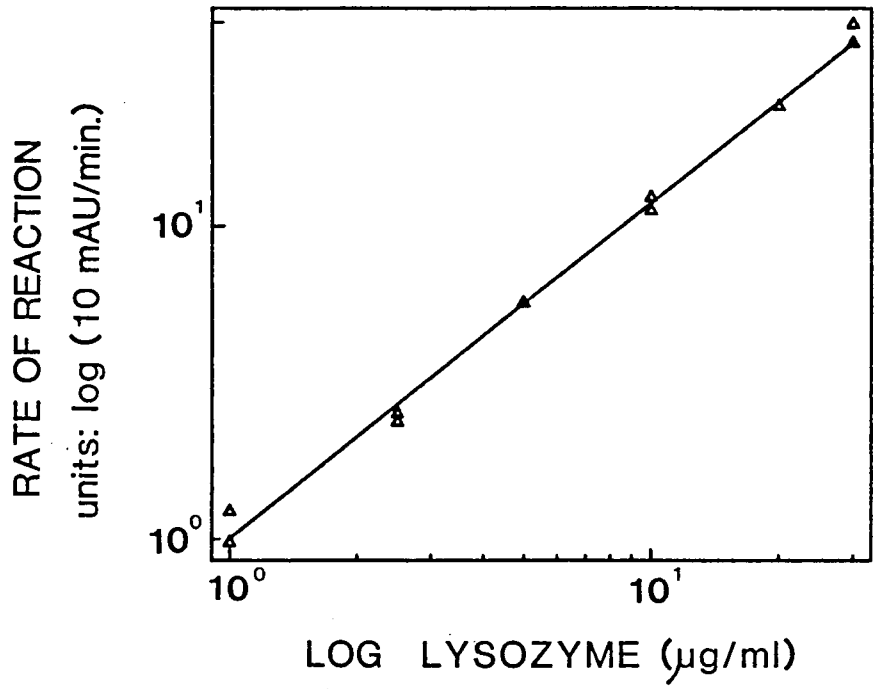


Fig 23 : Log-log linear regression : standard curve rate of decrease in turbidity of substrate suspension vs lysozyme concentration

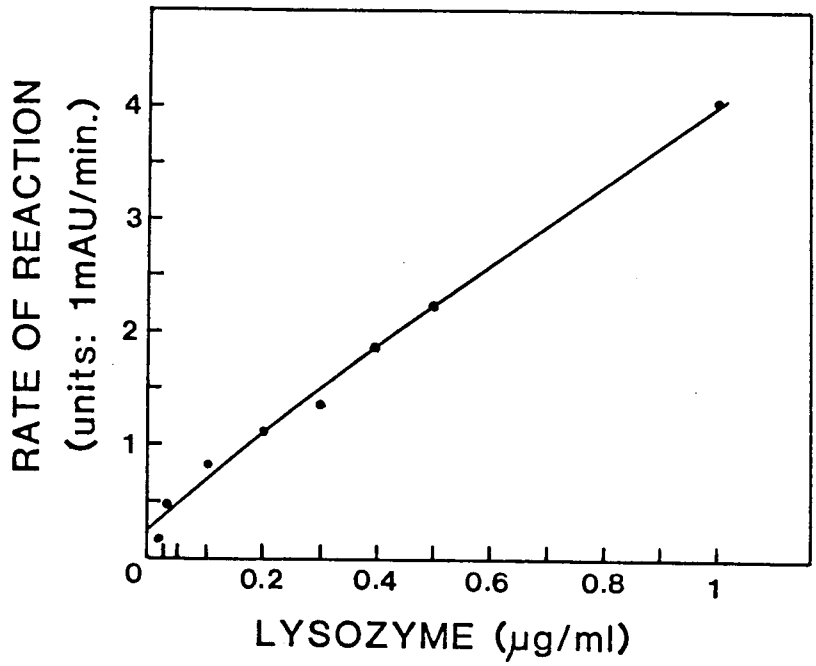


Fig 24: Exponential least squares regression : standard curve of rate of decrease in turbidity of substrate suspension vs lysozyme concentration

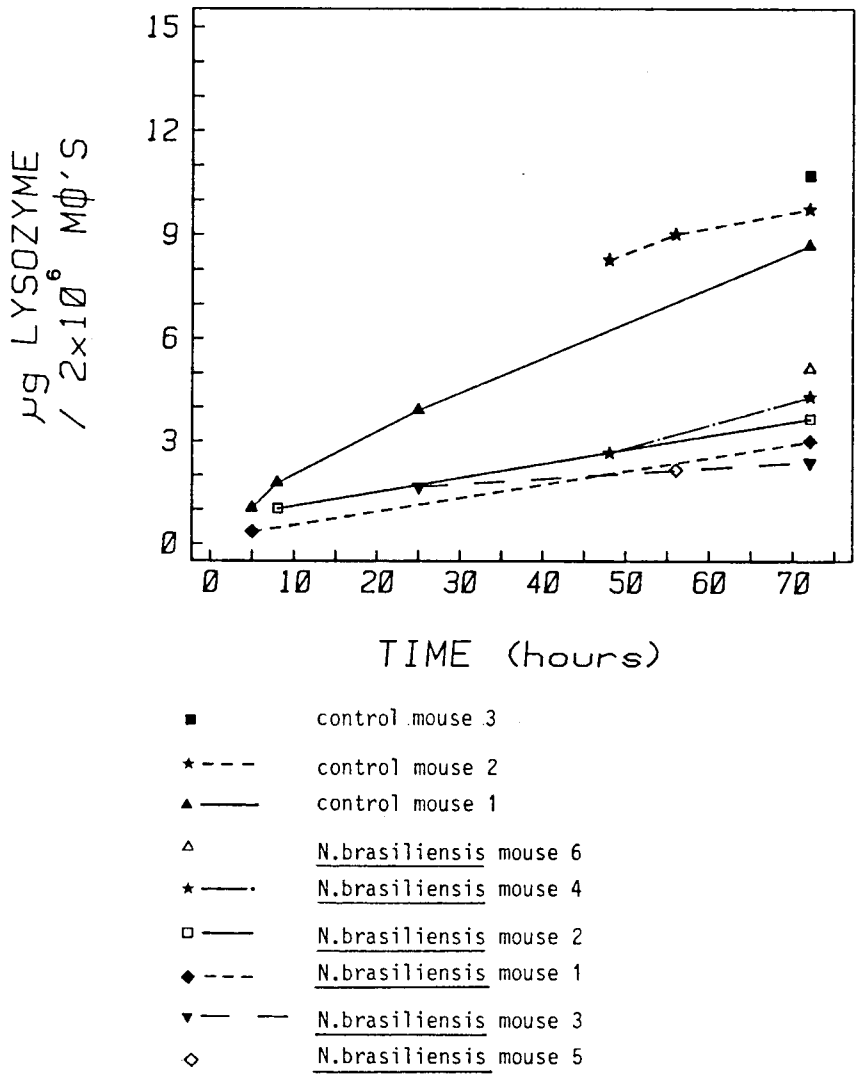


Fig 25 : Optimum incubation time.

Fig 26 : Cumulative lysozyme release by macrophages from mice inoculated 2 days previously with N.asteroides

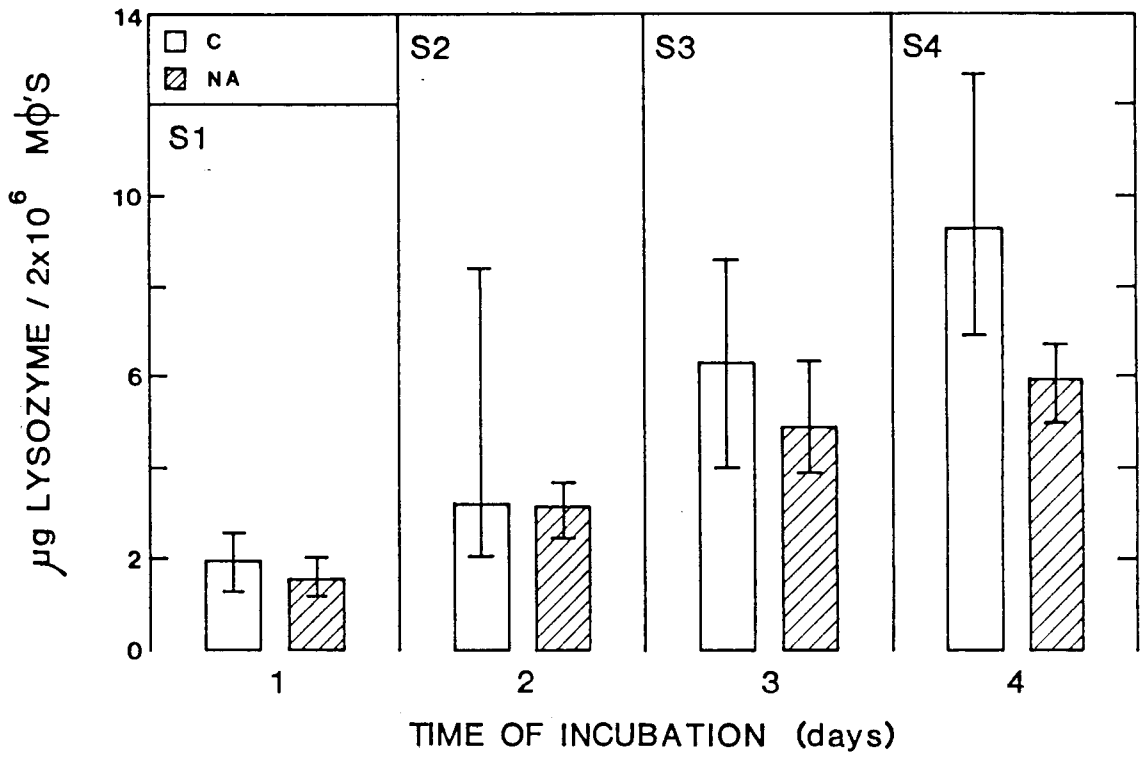


Fig 27 : Cumulative lysozyme release by macrophages from mice inoculated 2 days previously with N.brasiliensis

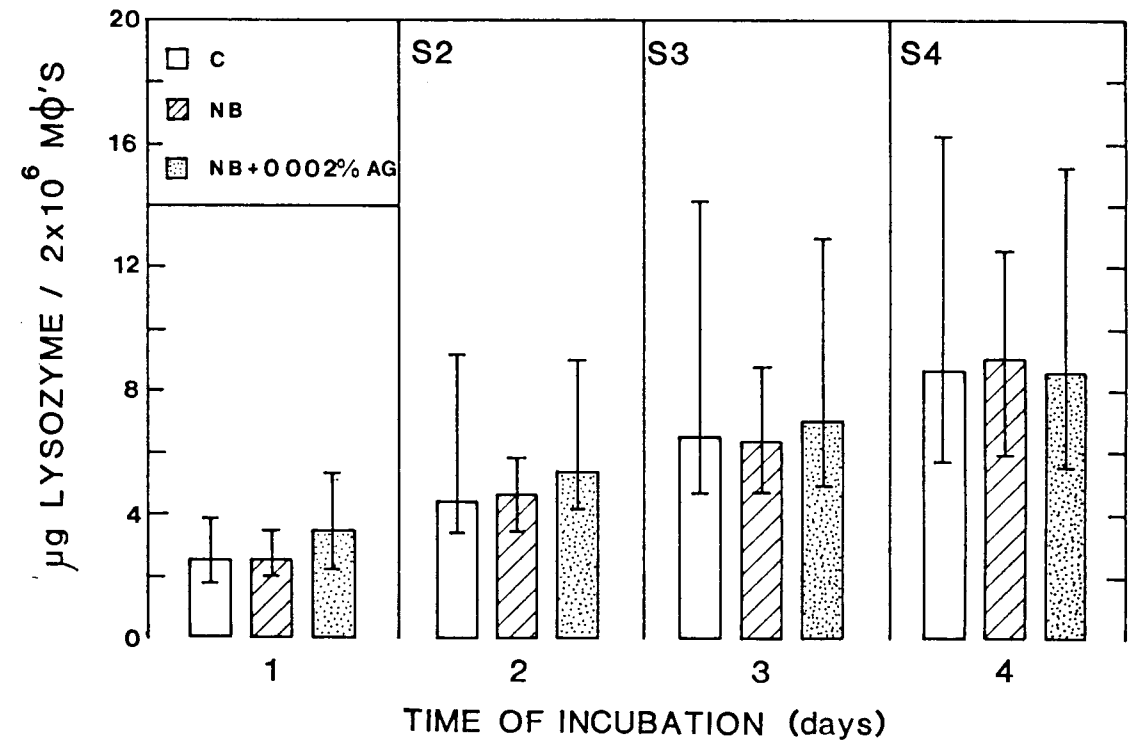


Fig 28 : Cumulative lysozyme release by macrophages from mice inoculated 7 days previously with N.asteroides

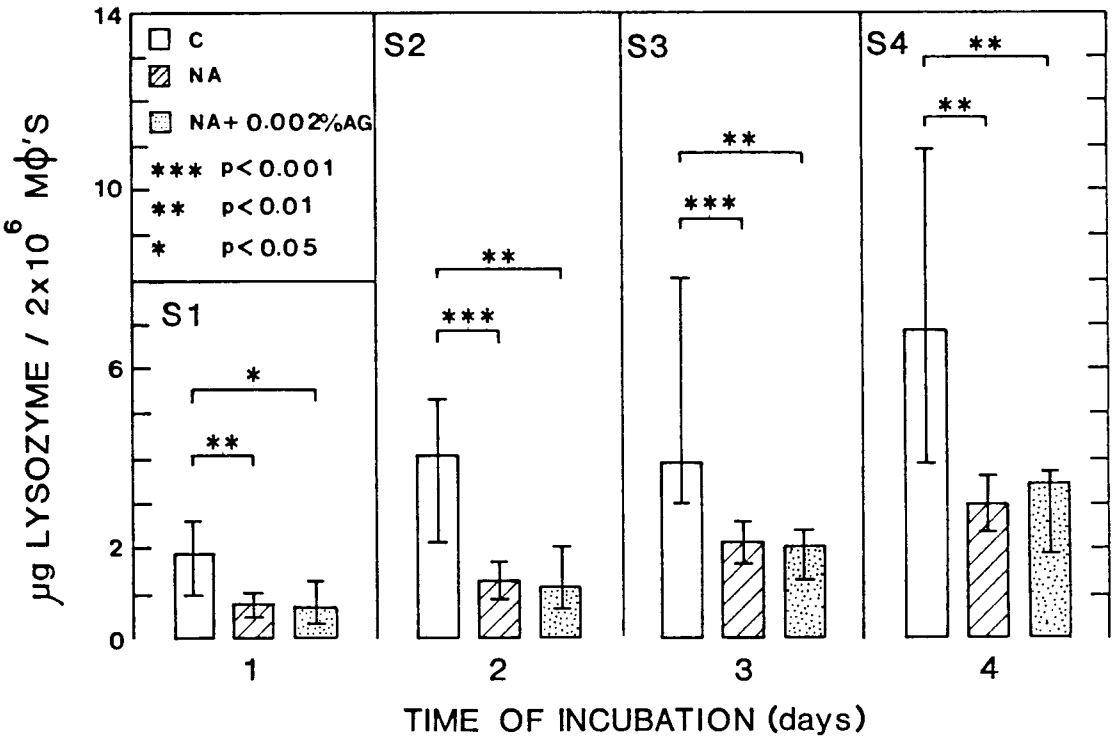


Fig 29 : Cumulative lysozyme release by macrophages from mice inoculated 7 days previously with N.brasiliensis

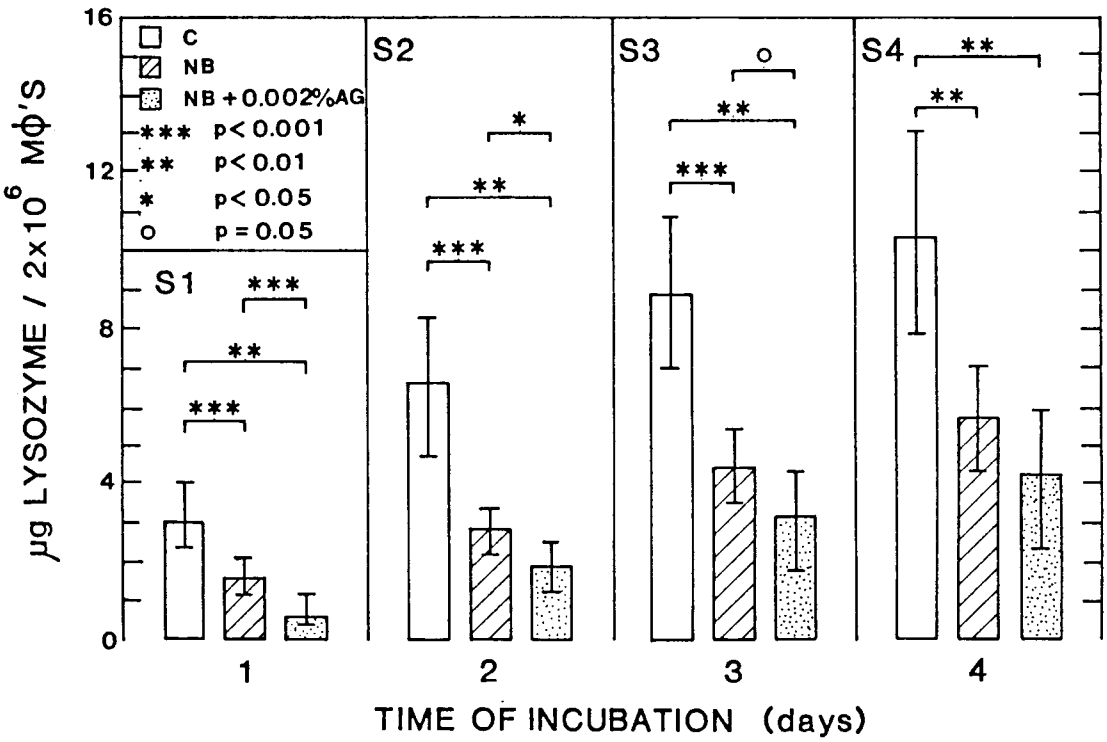


Fig 30 : Cumulative lysozyme release by macrophages from mice inoculated 13 days previously with N.asteroides

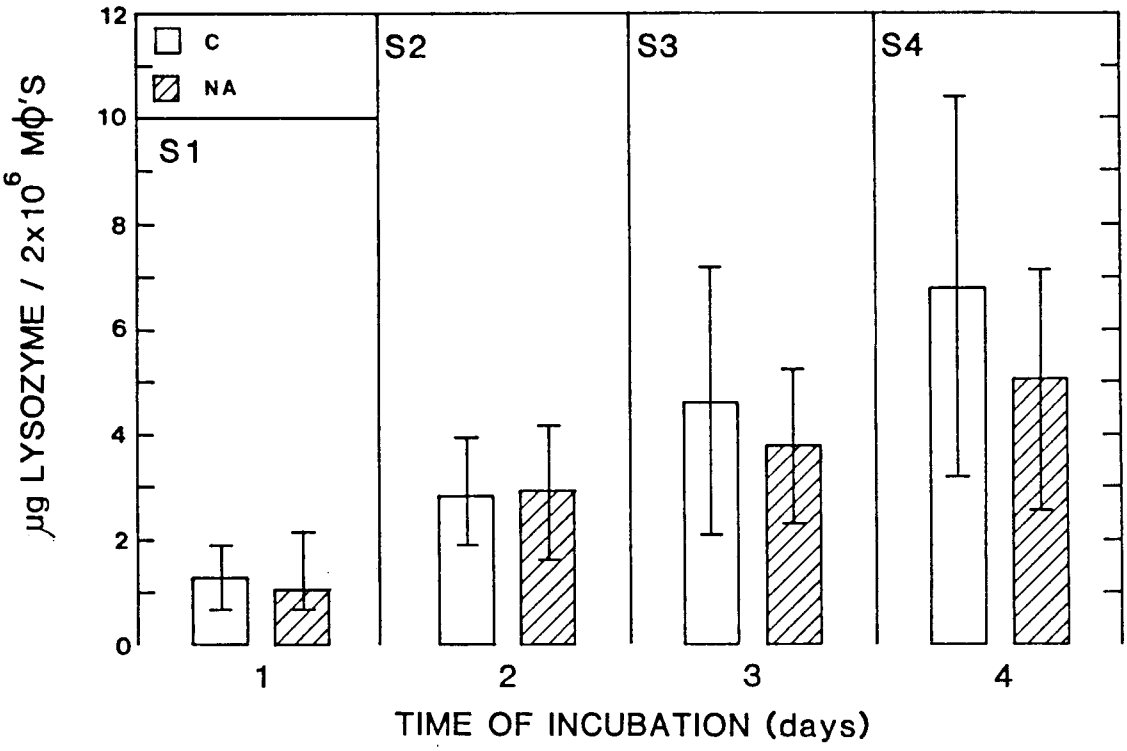


Fig 31 : Cumulative lysozyme release by macrophages from mice inoculated 13 days previously with N.brasiliensis

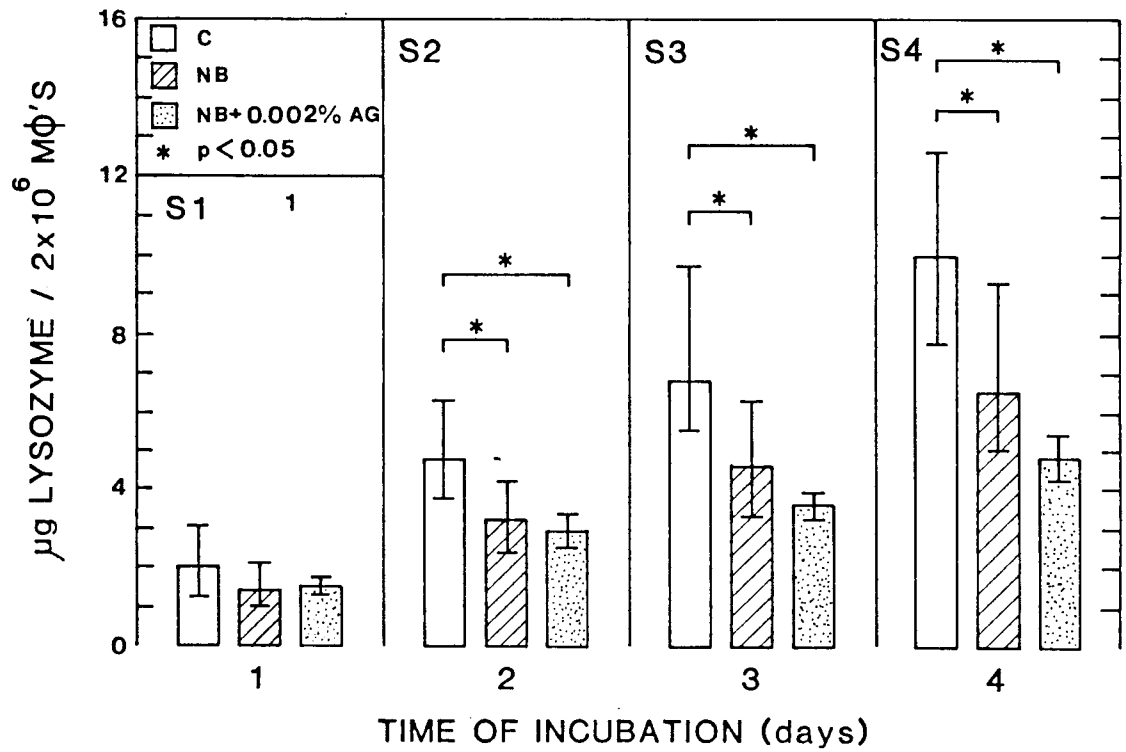


Fig 32 : Cumulative lysozyme release by macrophages from mice inoculated 21 days previously with N.asteroides

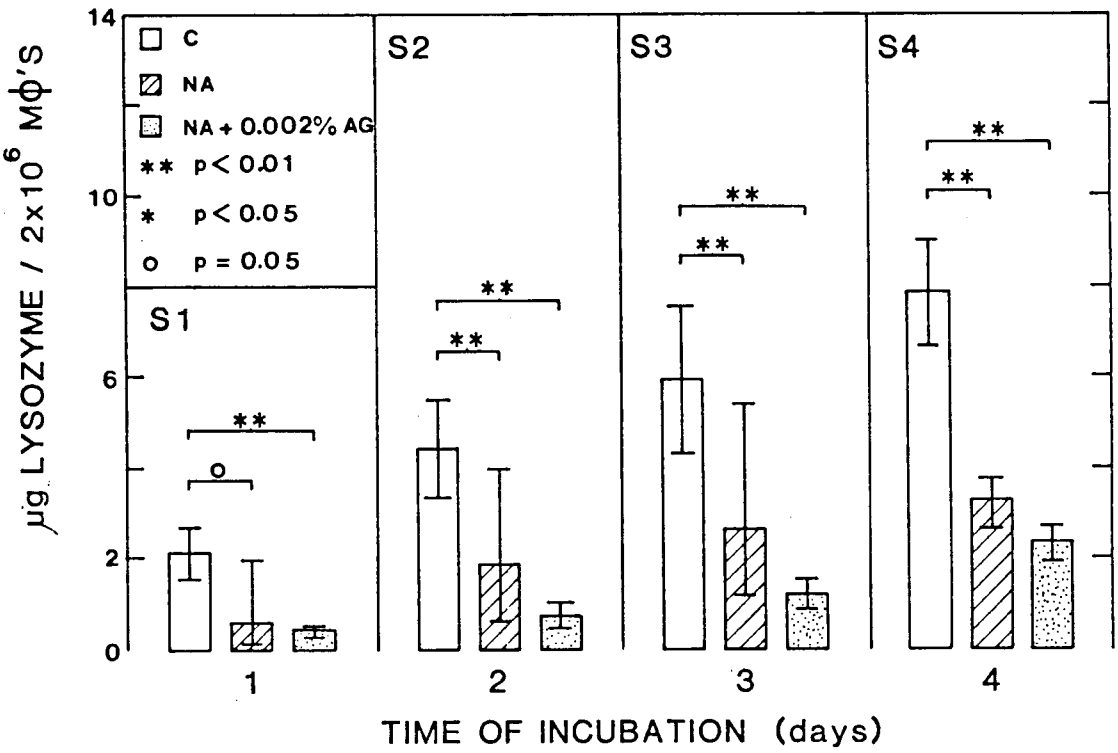
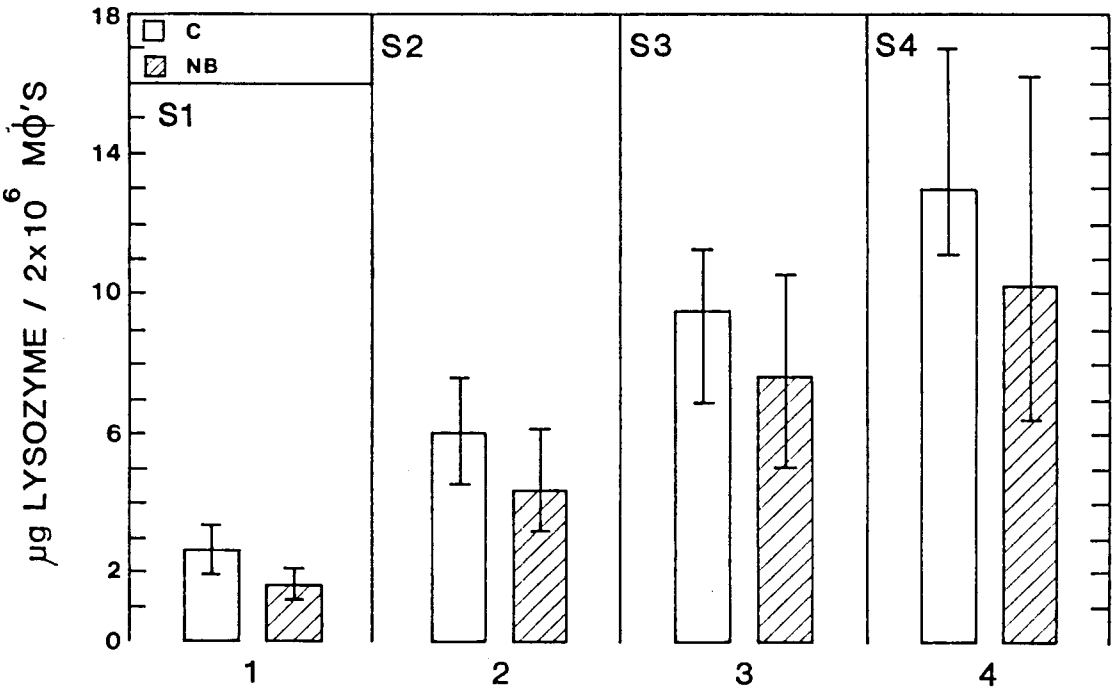


Fig 33 : Cumulative lysozyme release by macrophages from mice inoculated 21 days previously with N.brasiliensis



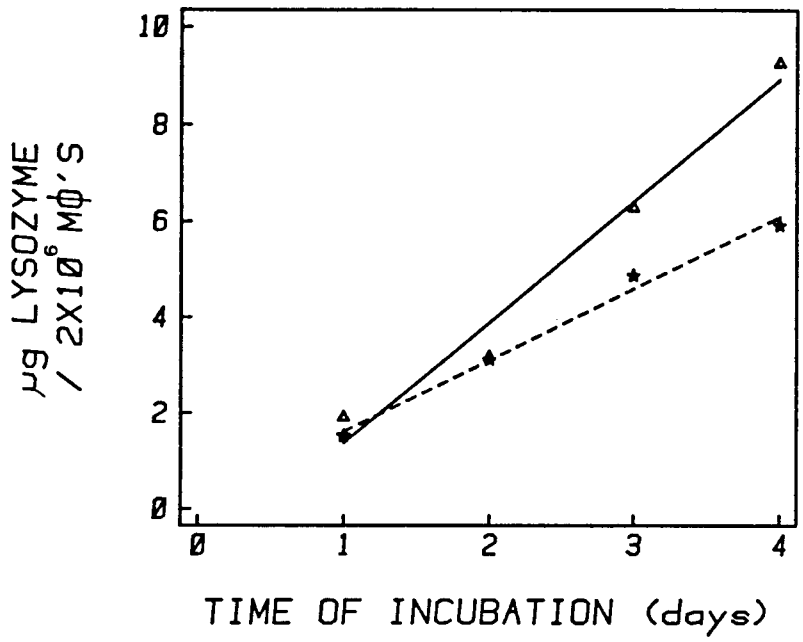


Fig 34 : Rate of lysozyme release by macrophages from mice inoculated 2 days previously with N.asteroides

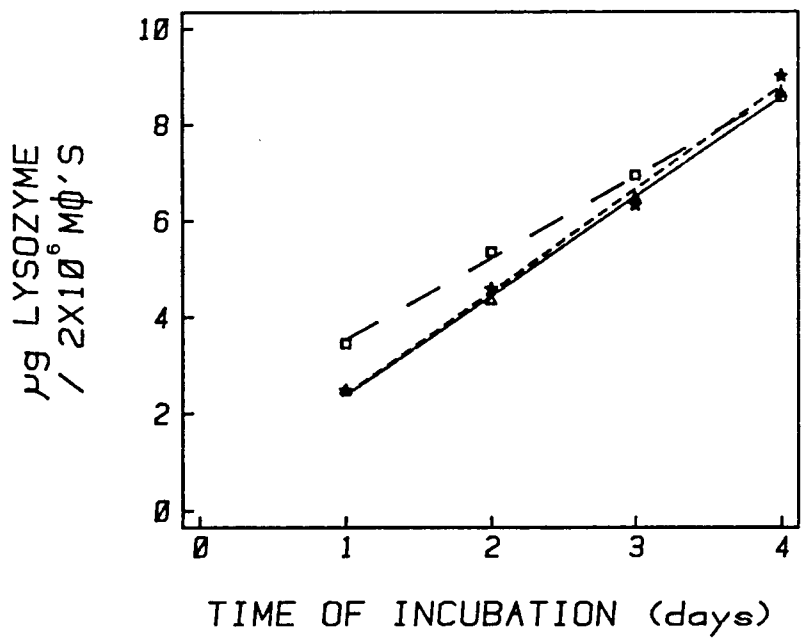


Fig 35 : Rate of lysozyme release by macrophages from mice inoculated 2 days previously with N.brasiliensis

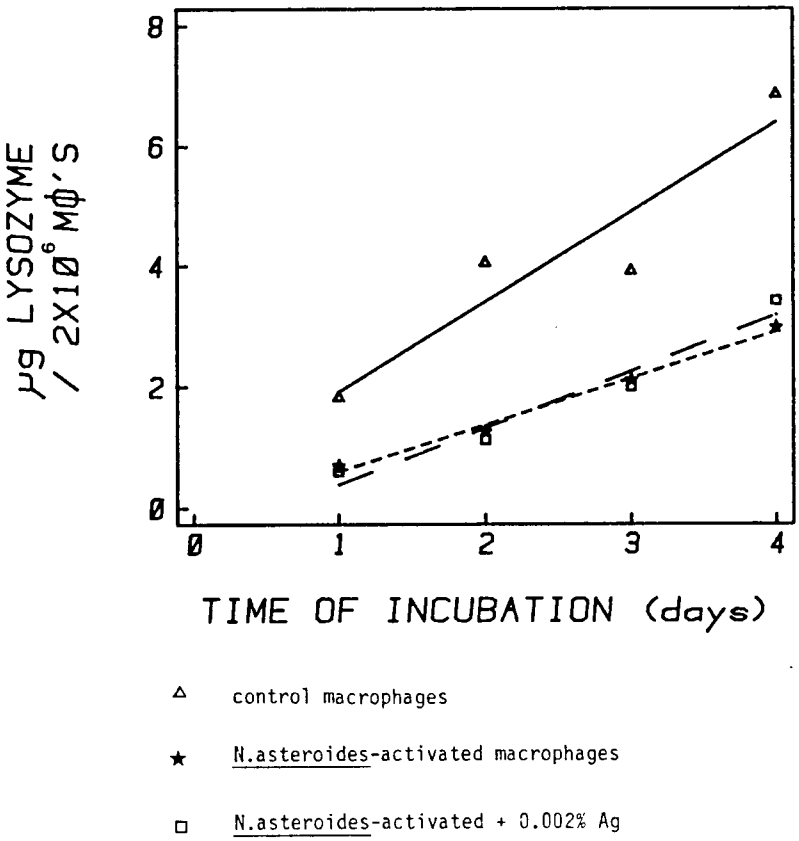


Fig 36 : Rate of lysozyme release by macrophages from mice inoculated 7 days previously with N.asteroides

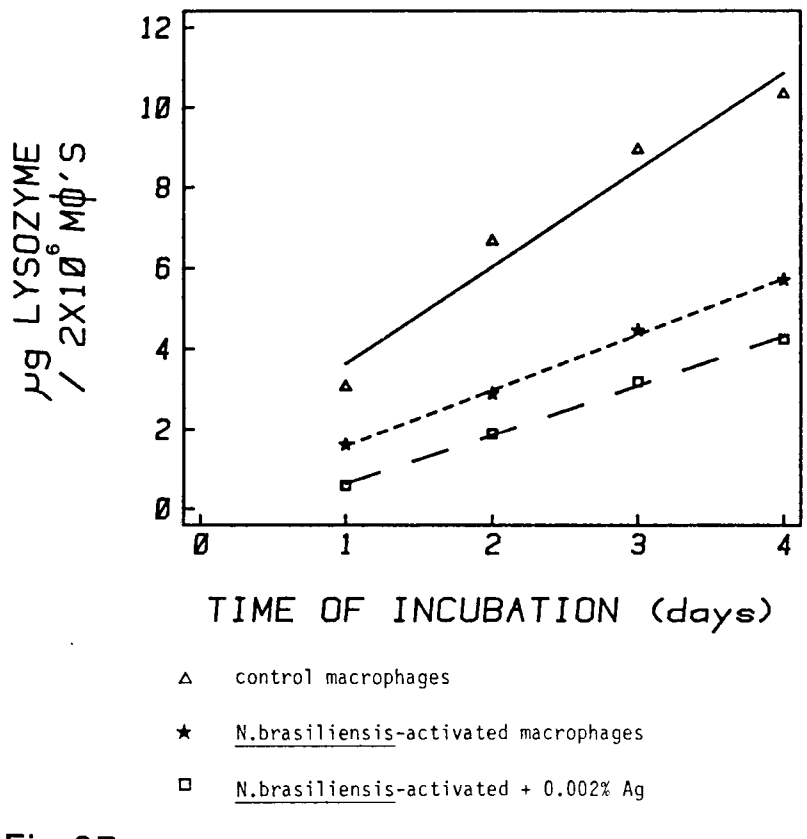


Fig 37 : Rate of lysozyme release of macrophages from mice inoculated 7 days previously with N.brasiliensis

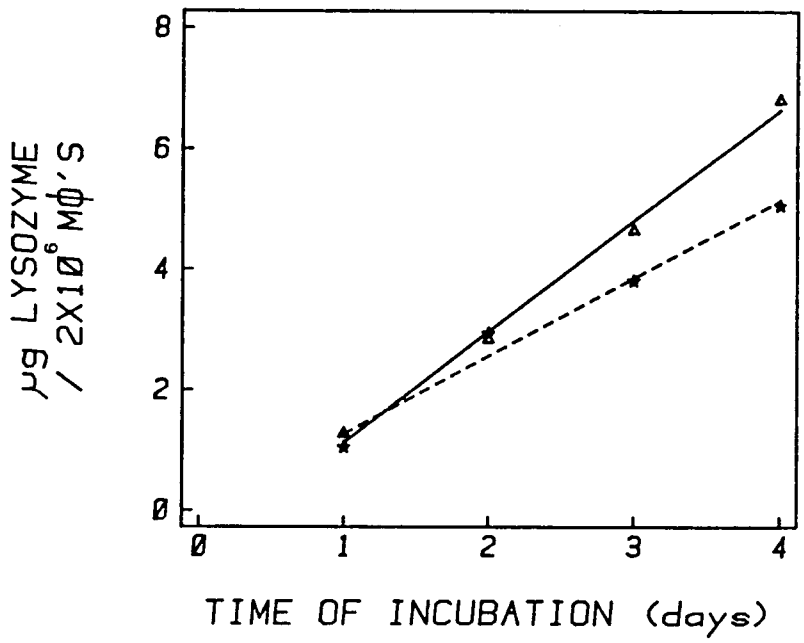


Fig 38 : Rate of lysozyme release by macrophages from mice inoculated 13 days previously with N.asteroides

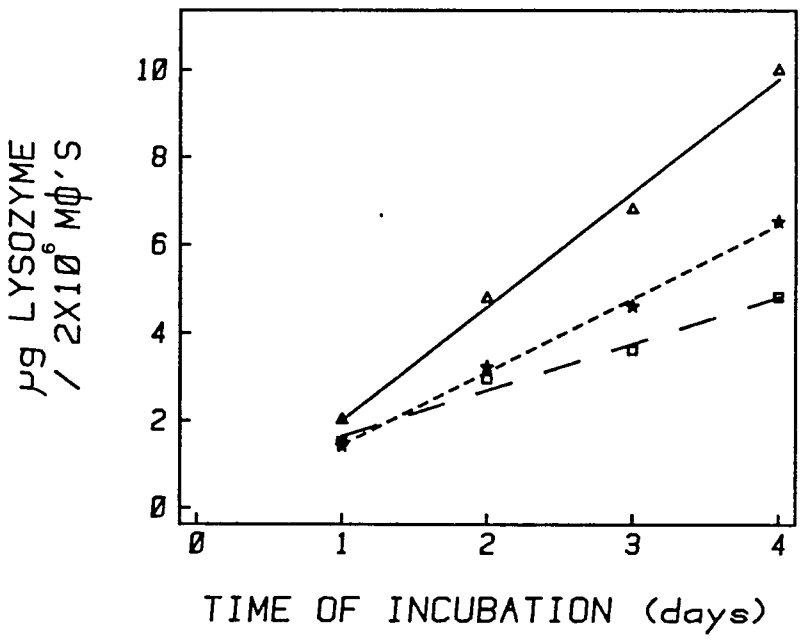


Fig 39 : Rate of lysozyme release by macrophages from mice inoculated 13 days previously with N.brasiliensis

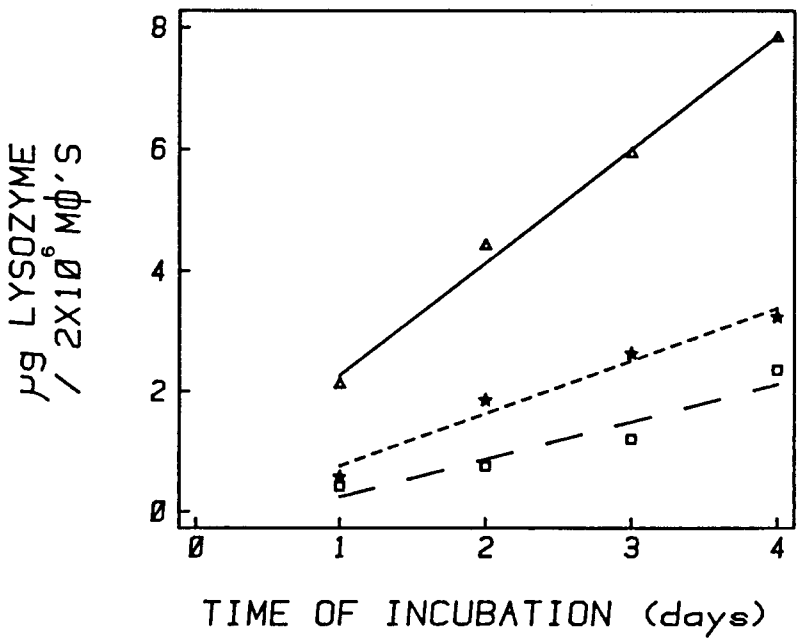


Fig 40 : Rate of lysozyme release by macrophages from mice inoculated 21 days previously with N.asteroides

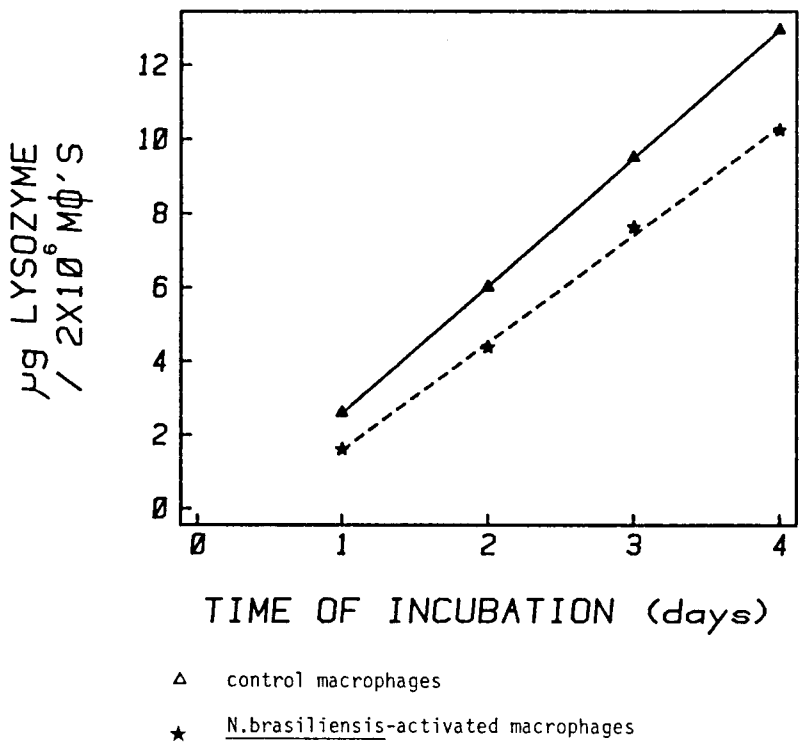


Fig 41: Rate of lysozyme release by macrophages from mice inoculated 21 days previously with N.brasiliensis

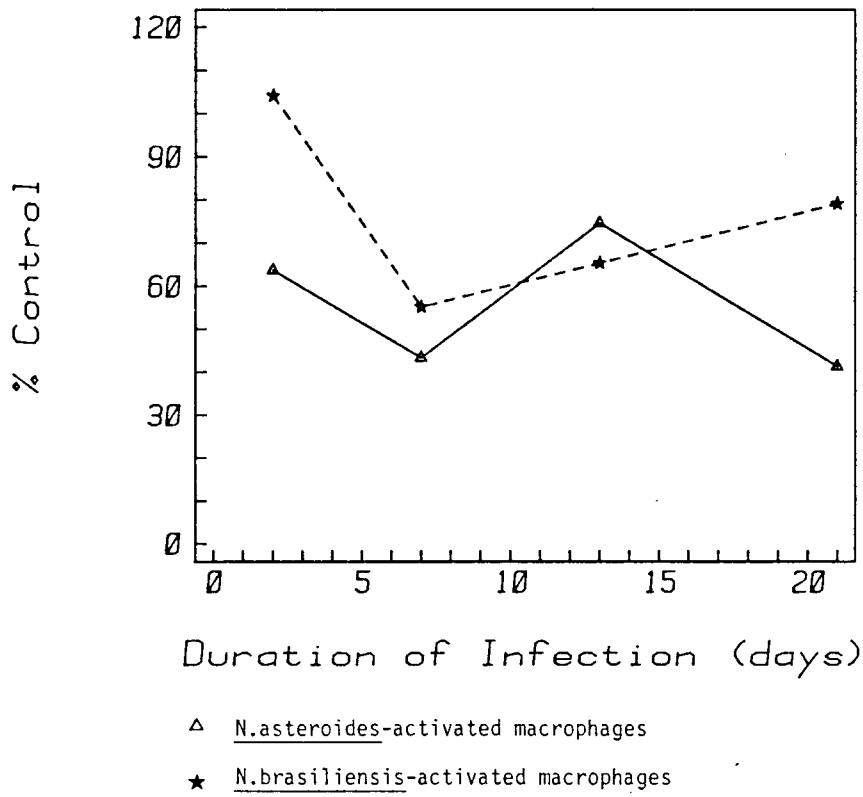


Fig 42 : Modulation of lysozyme release over the 21 day period of infection

4.6 TABLES

TABLE 73

Lysozyme production by 1 x 10^6 macrophages (µg of lysozyme)

13d Nb	S1 day 1	S2 day 2	S3 day 3	S4 day 4	S5 day 5	S6 day 6
C	2	4.5	9.25	10.25	11.75	13
Nb	0.5	1.5	3.25	4.5	5.75	7

KEY: C = 1 x 10^6 Control macrophages
Nb = 1 x 10^6 N.brasiliensis-activated macrophages
13d Nb = mice inoculated 13 days previously with N.brasiliensis

TABLE 74

Lysozyme production by 3 x 10^6 macrophages (µg of lysozyme)

13d Nb	S1	S2	S3	S4	S5	S6	S7
C	0.018	0.395	0.771	1.52	2.26	3.44	4.39
	0.376	1.281	2.186	2.93	4.12	5.8	6.53
Nb	0.376	0.694	0.896	1.28	2.99	4.68	5.4
	0.083	0.168	1.004	1.39	1.77	3.45	4.18
	0.083	0.104	0.544	2.26	2.39	4.07	5.25
	0.083	0.46	0.714	1.9	2.65	4.33	5.51
	0.083	0.46	2.15	2.53	2.92	5.66	6.38
	0.083	0.99	1.72	2.1	3.29	4.97	

KEY: C = 3 x 10^6 Control macrophages
Nb = 3 x 10^6 N.brasiliensis-activated macrophages
13d Nb = mice inoculated 13 days previously with N.brasiliensis

TABLE 75

Optimum time of incubation.

7d Nb	5 hrs	8hrs	25 hrs	48hrs	56 hrs	72 hrs
C1	1	1.738	3.878			8.658
C2				8.25	8.988	9.726
C3						10.7
Nb 1	0.33					2.97
Nb 2		1				3.64
Nb 3			1.65			2.388
Nb 4				2.64		4.29
Nb 5					2.14	
Nb 6						5.12

KEY: C = Control macrophages
Nb = N.brasiliensis-activated macrophages

Macrophages were harvested from control and N.brasiliensis inoculated mice 7 days after inoculation. 3 replicate cultures of control macrophages and 6 replicate cultures of activated macrophages were prepared as described previously. Conditioned medium was collected from the replicate cultures at different times (as shown in the tables) over the 72 hour incubation period and assayed for lysozyme activity. After collection of the CM, cultures were refed and returned to the incubator. The experiment was terminated after 72 hours and the cumulative lysozyme release determined.

Lysozyme accumulated in the medium over the 72 hour incubation period irrespective of time of incubation between sampling and it was decided that a 24 hourly sampling interval would be employed.

TABLE 76

Lysozyme release from 2 x 10^6 macrophages over an 8 day incubation period. Mice were inoculated with N.brasiliensis organisms or normal saline 13 days prior to sacrifice. (Micrograms of lysozyme)

2d Nb	S1	S2	S3	S4	S5	S6	S7	S8
C n=1	2.0	4.75	9.75	14.0	18.75	20.75	23.0	26.0
Nb	2.0	6.0	10.75	14.5	19.5	21.5	24.5	27.5
n=3	3.0	7.75	14.25	19.75	25.25	28.0	30.5	33.0
	2.25	5.0	9.0	14.25	20.0	23.0	25.5	28.0
x	2.417	6.25	11.33	16.17	21.58	24.17	26.83	29.5
sd	0.52	1.39	2.67	3.10	3.18	3.4	3.21	3.04

KEY: C = 2 x 10^6 Control macrophages
Nb = 2 x 10^6 N.brasiliensis-activated macrophages
x = Mean
sd = Standard deviation
n = no. of replicates

TABLE 77

2 day N.asteroides infection : accumulation of lysozyme in conditioned medium over 4 days in culture. Experimental series "A". Data from 4 replicate experiments for lysozyme released by control and N.asteroides-activated macrophages and 1 experiment for enzyme released by antigen-exposed macrophages. CM assayed at daily intervals.
(Data expressed as micrograms of lysozyme/$2x10^6$ macrophages)

S.int / Inc. time	E	S1 1 day	S2 2 days	S3 3 days	S4 4 days
C	1	1.28	2.54	4.31	2.53
		1.28	2.88	2.08	
		0.99	1.61		
	2	1.88			
	3a	1.91	3.24	5.75	9.02
		1.91	2.95	4.28	5.9
	4a	3.2	7.24	10.46	12.64
		3.2	9.47	13.41	15.97
Na	1	1.43	2.52	3.29	4.14
		1.28	2.71		
		1.28	2.71		
	2	0.85	3.28	4.88	5.99
		2.19	2.52	5.05	6.33
		1.43	4.6	6.03	7.91
		2.82	3.51	5.85	6.70
		1.73			
		0.85			
	3a	2.96	3.86	5.62	6.39
		1.76	3.82	4.86	5.76
		1.91	3.82	5.0	6.04
	4a	0.81	1.46	2.69	3.89
		0.81	1.62	3	4.09
Na+ 0.002% Antigen in vitro	4a	0.65	1.46	3.64	4.49
		0.81	1.79	2.72	4.41

KEY: S.int = Sampling interval
Inc.time = Incubation time
E = Experiment number
C = Control macrophages
Na = N.asteroides-activated macrophages

TABLE 78

2 day N.asteroides infection : specific activity of cumulative lysozyme released.

MØ type	E	F	Cumulative lysozyme released (µg)	Specific activity (µg per mg of cell protein)
C	3a	S4	9.Ø2	58.3
			5.9	28.12
	4a	S4	12.64	49.51
			15.97	58.23
Na	3a	S4	6.39	17.26
			5.76	12.63
			6.Ø4	21.61
	4a	S4	3.89	6.24
			4.Ø9	6.89
Na+ Ø.ØØ2% Antigen in vitro	4a	S4	4.49	2Ø.58
			4.41	8.3

KEY: E = Experiment number
F = Final sample

TABLE 79

2 day N.asteroides infection : Lysozyme production over 3 days in culture : % secretion (Lysozyme expressed as µg lysozyme per 2 x 10^6 macrophages). Experimental series "B".

		S1				S2				S3			
	E	a	b	c	d	a	b	c	d	a	b	c	d
C	3b	1.33	Ø.16	1.49	89.32	3.24	Ø.1Ø	3.34	96.9	4.71	Ø.15	4.86	96.99
						3.25	Ø.48	3.73	87.1	5.Ø1	Ø.Ø6	5.Ø7	98.83
	4b	3.2	Ø.23	3.43	93.2	6.14	Ø.4Ø	6.54	93.88	14.Ø6	Ø.61	14.67	95.83
		4.64	Ø.51	5.15	9Ø.Ø7	6.67	Ø.89	7.56	88.21	9.67	Ø.54	1Ø.21	94.67
Na	3b	1.91	Ø.17	2.Ø8	91.98	2.95	Ø.Ø4	2.99	98.75	4.71	Ø.17	4.88	96.5
		1.62	Ø.13	1.75	92.85	2.66	Ø.75	3.41	77.98	4.86	Ø.Ø9	4.95	98.2
						3.24	Ø.16	3.4Ø	95.3	3.69	Ø.19	3.88	95.Ø
	4b	Ø.51	Ø.Ø6	Ø.57	88.89	1.66	Ø.14	1.8Ø	92.29	4.41	Ø.24	4.65	94.9
		Ø.65	Ø.24	Ø.89	73.1	1.32	Ø.15	1.47	89.9	3.64	Ø.26	3.9Ø	93.2
Na**	4b	Ø.27	Ø.23	Ø.5Ø	54.23	1.96	Ø.Ø9	2.Ø5	95.5	3.33	Ø.31	3.64	91.6
		Ø.65	Ø.24	Ø.89	73.35	1.72	Ø.18	1.9Ø	9Ø.46	3.47	Ø.Ø3	3.5Ø	99.1

KEY: a = extracellular lysozyme; b = intracellular lysozyme; c = total lysozyme ; d = % secreted
Na** = N.asteroides-activated macrophages + Ø.ØØ2% antigen in vitro

TABLE 80

Specific activity of lysozyme produced by macrophages over 3 days in culture (Lysozyme expressed as μg of lysozyme per mg of cell protein).

		S1			S2			S3		
	E	a	b	c	a	b	c	a	b	c
C	3b	6.16	0.74	6.90	10.32	0.33	10.65	17.5	0.54	18.04
					18.09	2.68	20.77	26.49	0.32	26.81
	4b	9.63	0.70	10.33	17.28	1.13	18.41	50.16	2.18	52.35
		10.04	1.11	11.15	19.90	2.66	22.56	29.73	1.67	31.40
Na	3b	6.90	0.60	7.50	12.38	0.16	12.54	17.11	0.61	17.72
		5.23	0.40	5.63	10.93	3.09	24.01	22.7	0.43	23.13
					12.73	0.63	13.36	16.57	0.86	17.43
	4b	2.07	0.26	2.33	5.99	0.05	6.49	16.58	0.89	17.47
		3.05	1.12	4.17	3.48	0.64	4.11	18.37	0.16	38.52
Na**	4b	0.92	0.78	1.70	9.93	0.47	10.40	12.71	1.18	13.89
		3.26	1.19	4.45	5.44	0.57	6.01	13.14	0.12	13.26

KEY: E = Experiment number
a = Extracellular lysozyme
b = Intracellular lysozyme
c = Total lysozyme
C = Control macrophages
Na = N.asteroides-activated macrophages
Na** = N.asteroides-activated macrophages + 0.002% antigen in vitro

TABLE 81

2 day N.brasiliensis infection : Accumulation of lysozyme in conditioned medium over 4 days in culture. Experimental series "A". Data from 8 replicate experiments in 3 of which N.brasiliensis -activated macrophages were exposed to antigen in vitro. Lysozyme expressed as µg of lysozyme per 2 x 10^6 macrophages.

S.int / Inc.time	E	S1 1 day	S2 2 days	S3 3 days	S4 4 days
C	2	2.0	4.75	9.75	14.0
	3	2.47	4.48	6.49	7.84
	4	1.74	4.27	6.53	7.46
	5	1.53	3.08	4.07	4.52
		1.66	3.79	5.35	6.77
		1.88	3.76	5.31	6.16
	6	1.56	2.43	2.88	4.04
		2.48	2.95	3.15	3.71
		1.56	1.89	3.17	3.58
	7a	5.6	14.06	22.13	25.53
		10.9	17.87	25.2	28.6
	8a	2.56	4.91	6.56	10.5
		3.33	5.02	6.93	9.88
		3.85	5.54	7.32	11.01
Nb	1	0.90	2.16	3.85	
		0.83	1.44	1.82	
	2	2.0	6.0	10.75	14.5
		3.0	7.75	14.25	19.75
		2.25	5.0	9.0	14.25
	3	2.59	3.94	6.18	7.42
		2.47	4.25	5.81	7.26
		2.47	3.82	4.76	6.21
		2.47			
		2.47			
	4	0.82	2.32	3.58	4.01
		1.04	3.0	4.04	4.66
		1.38	2.64	3.46	4.08
		1.26			
	5	1.94	2.97	4.72	5.18
		1.8	3.42	4.0	4.45
		2.07	3.73	4.38	5.1
		1.8	3.35	4.38	4.71
	6	3.04	3.76	5.04	6.33
		2.34	2.92	3.64	4.59
	7a	6.97	13.24	19.17	22.03
		6.27	11.87	18.14	20.32
	8a	5.08	7.09	8.74	11.91
		4.32	6.5	8.28	10.74
		5.47	7.48	8.64	11.47
		4.32	5.85	7.89	11.71
Nb**	5	2.07	4.47	5.57	5.9
		1.94	4.92	6.23	6.68
		2.49	3.84	4.37	5.09
	7a	6.27	11.87	17.47	19.82
	8a	3.4	5.12	6.65	9.23
		4.32	6.18	8.22	10.44
		3.22	5.84	7.75	10.45

KEY : S.int = Sampling interval
Inc.time = Incubation time
E = Experiment number
Nb = N.brasiliensis-activated macrophages
Nb** = N.brasiliensis-activated macrophages + 0.002% antigen in vitro

TABLE 82

2 day N.brasiliensis infection : specific activity of cumulative lysozyme released.

MØ Type	E	F	Cumulative lysozyme released (µg)	Specific activity (µg per mg of cell protein)
C	5	S6	10.13	52.59
			9.88	61.48
			10.48	79.1
	6	S6	6.39	48.7
			5.17	31.75
			5.42	42.27
	7a	S4	25.53	82.81
			28.6	98.87
	8a	S4	10.5	68.09
			9.88	47.35
			11.01	61.43
Nb	5	S6	9.76	72.3
			9.41	86.1
			8.23	65.4
			7.28	64.4
	6	S6	12.81	73.15
			9.34	44.7
	7a	S4	22.03	83.99
			20.32	70.49
	8a	S4	11.91	61.72
			10.74	82.62
			11.47	53.0
			11.71	64.09
Nb + 0.002% Ag in vitro	5	S6	7.98	60.18
			9.53	95.35
			11.15	85.7
	7a	S4	19.82	58.43
	8a	S4	9.23	49.13
			10.44	68.87
			10.45	53.88

KEY: E = Experiment number
F = Final sample

TABLE 83

2 day N.brasiliensis infection : Lysozyme production over 3 days in culture : % secretion (Lysozyme expressed as µg lysozyme per 2 x 10^6 macrophages). Experimental series "B".

		S1				S2				S3			
	E	a	b	c	d	a	b	c	d	a	b	c	d
C	7b	6.97	Ø.87	7.84	88.9	12.9	Ø.95	13.85	93.15	21.Ø1	Ø.67	21.68	96.9
						15.4	1.15	16.55	93.Ø5	28.87	Ø.69	29.56	97.67
	8b	4.25	Ø.4Ø	4.65	91.3	6.19	Ø.36	6.55	94.6	6.57	Ø.2Ø	6.77	97.Ø9
		3.96	Ø.56	4.32	86.97	8.Ø7	Ø.46	8.53	94.6	6.Ø	Ø.19	6.19	96.95
		3.94	Ø.81	4.75	82.88	5.38	Ø.46	5.84	92.Ø	7.85	Ø.2Ø	8.Ø5	97.5
Nb	7b	5.27	Ø.68	5.95	88.58	14.66	Ø.45	15.11	97.Ø2	19.94	Ø.39	2Ø.33	98.1
	8b	3.58	Ø.57	4.15	86.19	5.92	Ø.22	6.14	96.4	8.71	Ø.3Ø	9.Ø1	96.67
		4.7	Ø.16	4.86	96.6	5.41	Ø.23	5.64	95.9	8.43	Ø.32	8.75	96.36
		3.Ø4	Ø.22	3.26	93.3	6.52	Ø.16	6.68	97.7	7.93	Ø.19	8.12	97.66
										7.93	Ø.21	8.15	97.3
Nb**	7b	5.6	Ø.98	6.58	85.Ø7	9.68	Ø.44	1Ø.12	95.66	12.85	Ø.15	13.Ø	98.85
	8b	3.58	Ø.5Ø	4.Ø8	87.7	5.Ø5	Ø.34	5.39	93.7	8.21	Ø.24	8.45	97.19
		4.13	Ø.1Ø	4.23	97.65	5.66	Ø.28	5.94	95.4	8.8	Ø.21	9.Ø1	97.64
		3.Ø4	Ø.81	3.85	78.9	5.89	Ø.13	6.Ø2	97.8	8.58	Ø.23	8.81	97.42

KEY: a = extracellular lysozyme; b = intracellular lysozyme; c = total lysozyme ; d = % secreted
Nb** = N.brasiliensis-activated macrophages + Ø.ØØ2% antigen in vitro

TABLE 84

2 day N.brasiliensis infection : specific activity of lysozyme produced by macrophages over 3 days in culture. (Lysozyme expressed as µg of lysozyme/mg of cell protein)

		S1			S2			S3		
	E	a	b	c	a	b	c	a	b	c
C	7b	11.33	1.41	12.74	25.95	1.91	27.86	46.67	1.49	48.61
					27.11	2.03	29.14	71.78	1.71	73.49
	8b	13.64	1.49	17.13	30.42	1.75	32.18	40.14	1.20	41.34
		13.37	2.00	15.37	31.48	1.80	33.28	38.27	1.20	39.47
		11.47	2.37	13.84	25.47	2.18	30.65	47.45	1.22	48.67
Nb	7b	12.14	1.57	13.70	39.7	1.22	40.92	74.88	1.45	76.33
	8b	17.02	2.73	19.75	27.69	1.03	28.72	45.32	1.57	46.89
		36.4	1.27	37.67	27.89	1.18	29.09	29.52	1.12	30.64
		13.83	1.0	14.83	35.35	0.84	36.19	44.89	1.07	45.97
								43.20	1.19	44.39
Nb**	7b	14.30	2.51	16.81	28.45	1.29	29.74	41.82	0.49	42.31
	8b	14.68	2.05	17.72	20.33	1.37	21.70	45.58	1.32	46.90
		25.23	0.61	25.85	28.06	1.37	29.43	48.17	1.16	49.33
		9.27	2.48	11.75	26.47	0.59	27.06	47.46	1.26	48.71

KEY: E = Experiment number
a = Extracellular lysozyme
b = Intracellular lysozyme
c = Total lysozyme
C = Control macrophages
Nb = N.brasiliensis-activated macrophages
Nb** = N.brasiliensis-activated macrophages + 0.002% antigen in vitro

TABLE 85

7 day N.asteroides infection : Accumulation of lysozyme in conditioned medium over 4 days in culture. Experimental series "A". Data from 10 replicate experiments, in 4 of which N.asteroides -activated macrophages were exposed to antigen in vitro. Lysozyme expressed as µg of lysozyme per 2 x 10^6 macrophages.

S.int Inc.time	E	S1 1 day	S2 2 days	S3 3 days	S4 4 days
	1	4.6	5.88		
		4.34	8.18		
	2	0.91	2.75	4.07	4.66
		1.28	2.28	3.55	3.98
		1.15	2.42	4	4.52
C					
	3	0.03	1.01	2.1	2.5
		0.22	2.06		
	4	0.84	2.2	2.62	3.71
		0.88			
	5	2.36	7.49		
		3.74			
	6	1.53	2.48	3.34	9.23
	9a	1.01	1.52	3.36	4.39
		0.69	1.84	2.88	3.91
	10a	2.93	5.78	11.14	16.11
		2.93	7.99	12.7	17.15
		2.62	5.89	10.47	15.7
	2	0.67	0.92	1.06	1.49
		0.004	0.23	1.58	1.6
		0.67	0.78		
		0.44	0.62		
		1.28	1.42		
		0.004	0.44		
Na					
	4	0.34	1.2	1.87	2.52
		1.39	2.59	2.88	3.52
		0.12	0.66	1.37	2.01
		0.99	1.53	2.07	3.76
		0.51	1.59	1.92	3.48
		0.22	0.87	1.32	2.10
		0.91	1.55	1.62	2.17
		0.84	1.49	1.62	2.16
		0.51	1.16	1.32	1.87
		0.99	1.85	2.25	3.22
		0.22	0.87	1.57	2.54

TABLE 85 continued

	5	1.86			
	6	1.21	2.41	3.38	5.74
		Ø.71	4.16	4.91	5.86
		Ø.29	Ø.77	2.33	3.28
		1.21	1.63	2.7	3.65
	7	1.28	1.73	3.31	4.31
		2.19	3.47	4.75	5.74
		1.88	3.16		
	8	Ø.58	Ø.9	2.33	2.78
		Ø.45	2.3	4.18	5.Ø3
		Ø.72			
		Ø.72			
	9a	Ø.38	Ø.73	Ø.88	Ø.99
		Ø.38	Ø.65	1.Ø	1.11
		Ø.18	Ø.62	Ø.96	1.19
		Ø.28	Ø.55	Ø.7Ø	Ø.91
	1Øa	1.17	1.77	2.85	4.71
		Ø.44	Ø.94	2.13	3.54
		Ø.67	1.Ø7	2.Ø3	3.33
		Ø.79	1.39	2.91	3.88
	5	1.16	3.Ø5		
		2.36			
		1.38			
	6	Ø.53	Ø.78	1.86	3.24
		Ø.15	1.32	2.Ø7	3.45
		Ø.29			
Na**					
	9a	Ø.38	Ø.65	Ø.8	Ø.91
		Ø.18	Ø.32	Ø.67	Ø.71
	1Øa	Ø.67	1.16	2.35	3.87
		Ø.67	1.Ø7	2.Ø3	3.67
		Ø.55	1.6Ø	2.79	3.54
		Ø.44	1.15	2.23	3.64

KEY : S.int = Sampling interval
Inc.time = Incubation time
E = Experiment number
Na = N.asteroides-activated macrophages
Na ** = N.asteroides-activated macrophage cultures + Ø.ØØ2% antigen in vitro

TABLE 86

7 day N.asteroides infection : specific activity of cumulative lysozyme released.

MØ Type	E	F	Cumulative lysozyme released (µg)	Specific activity (µg per mg of cell protein)
	4	S4	3.73	27.58
C	9a	S4	4.39	9.83
			3.91	9.44
	10a	S4	16.11	67.89
			17.15	66.76
			15.7	53.02
	4	S4	2.52	21.69
			3.52	27.59
			2.01	13.38
			3.76	16.53
			3.48	20.36
			2.1	13.64
Na			2.16	18.3
			1.87	15.46
			3.22	20.56
			2.54	16.24
	9a	S4	0.99	6.42
			1.11	5.03
			1.19	7.38
			0.91	3.92
	10a	S4	4.71	21.62
			3.54	10.54
			3.33	8.77
			3.88	15.00
Na + 0.002% Ag in vitro	9a	S4	0.91	4.29
			0.71	2.77
	10a	S4	3.87	20.46
			3.67	19.78
			3.54	18.09
			3.64	19.06

KEY: E = Experiment number
F = Final sample

TABLE 87

7 day N.asteroides infection : Lysozyme production over 3 days in culture : % secretion (Lysozyme expressed as µg lysozyme per 2 x 10^6 macrophages). Experimental series "B".

	E	S1 a	b	c	d	S2 a	b	c	d	S3 a	b	c	d
C	9b	1.01	0.14	1.15	88	1.89	0.29	2.18	86.68	3.85	0.91	4.77	80.8
		0.69	0.34	1.03	67.3	2.43	0.60	3.03	80.14	3.57	0.25	3.83	93.3
	10b	2.46	1.22	3.69	66.67	6.78	1.45	8.23	82.4	11.96	0.92	12.88	92.9
		1.81	0.52	2.37	78.55	6.77	1.03	7.80	86.75	10.72	0.70	11.42	94.2
Na	9b	1.46	0.34	1.80	81.1	0.71	0.006	0.71	99.1	1.05	0.03	1.08	97.7
		0.58	0.03	0.61	95.4	0.48	0.12	0.60	83.5	0.80	0.06	0.87	92.8
		0.38	0.11	0.50	77.8	1.21	0.14	1.35	89.5	0.97	0	0.80	95.5
		0.48	0.14	0.62	76.9	0.65	0.07	0.72	90.07	0.77	0.36		
	10b	0.66	0.07	0.73	91.6	1.61	0.23	1.84	87.34	2.76	0.24	3.00	92.1
		0.66	0.07	0.73	91.4	1.50	0.06	1.56	96.5	3.19	0.20	3.39	94.2
		0.43	0.04	0.51	86.4	1.26	0.14	1.40	89.95	2.58	0.09	2.67	96.7
		0.66	0.07	0.73	91.6	1.15	0.29	1.43	79.99	2.35	0.20	2.55	92.3
Na**	9b	0.28	0.11	0.38	71.8	0.65	1.52	0.81	81.1	1.11	0		
		0.28	0.02	0.30	92.8	0.73	0.16	0.89	81.7	1.00	0.07	1.07	93.2
		0.38	0.03	0.41	87.5	0.73	0.02	0.75	97.66	0.72	0.04	0.74	95.6
	10b	0.79	0.01	0.80	98.21	2.08	0.12	2.20	89.95	2.34	0.03	2.37	98.9
		1.17	0.03	1.20	97.33	1.92	0.38	2.32	83.4	3.52	0.01	3.54	99.6
		1.17	0.08	1.24	93.98	1.65	0.30	1.94	84.6	2.33	0.12	2.46	95
		0.79	0.01	0.80	98.21	1.96	0.18	2.14	91.4	3.48	0.07	3.56	98.05

KEY: a = extracellular lysozyme; b = intracellular lysozyme; c = total lysozyme ; d = % secreted
Na** = N.asteroides-activated macrophages + 0.002% antigen in vitro

TABLE 88

7 day N.asteroides infection : specific activity of lysozyme produced by macrophages over 3 days in culture. (Lysozyme expressed as µg of lysozyme/mg of cell protein)

		S1			S2			S3		
	E	a	b	c	a	b	c	a	b	c
C	9b	3.77	0.52	4.29	7.23	1.11	8.35	16.41	3.88	20.29
		2.88	1.4	4.28	10.05	2.49	12.54	47.28	3.37	50.65
	10b	5.01	2.50	7.51	19.59	4.18	23.77	53.15	4.09	57.24
		3.40	0.93	4.33	21.15	3.23	24.38	41.99	2.58	44.57
Na	9b	9.99	2.33	12.32	3.99	0.04	4.03	7.81	0.19	8.00
		3.15	0.15	3.3	2.70	0.64	3.34	3.73	0.29	4.02
		2.44	0.69	3.13	2.01	0.83	7.85	4.29	0	0
		1.5	0.45	1.95	3.71	0.41	4.12	5.89	0.28	6.17
	10b	4.22	0.39	4.60	9.6	1.39	10.99	10.13	0.87	11.00
		4.57	0.43	5.00	8.3	0.3	8.6	17.14	1.1	18.24
		2.85	0.45	3.30	7.6	0.85	8.45	16.54	0.58	17.11
		4.20	0.38	4.58	6.36	1.59	7.95	16.53	1.4	17.93
Na**	9b	1.47	0.57	2.04	3.71	0.86	4.57	4.86	0	0
		1.04	0.08	1.12	3.97	0.89	4.86	4.27	0.31	4.58
		2.42	0.35	2.77	3.17	0.08	3.25	3.33	0.17	3.50
	10b	5.50	0.1	5.60	10.77	0.62	11.39	13.38	0.15	13.53
		8.39	0.23	8.62	14.05	2.79	16.84	17.71	0.06	17.78
		4.65	0.30	4.94	9.94	1.8	11.74	12.26	0.64	12.90
		4.88	0.09	4.97	12.49	1.17	13.66	16.19	0.32	16.51

KEY: E = Experiment number
a = Extracellular lysozyme
b = Intracellular lysozyme
c = Total lysozyme
C = Control macrophages
Na = N.asteroides-activated macrophages
Na** = N.asteroides-activated macrophages + 0.002% antigen in vitro

TABLE 89

7 day N.brasiliensis infection : Accumulation of lysozyme in conditioned medium over 4 days in culture. Experimental series "A". Data from 8 replicate experiments, in 5 of which N.brasiliensis -activated macrophages were exposed to antigen in vitro. Lysozyme expressed as µg of lysozyme per 2 x 10^6 macrophages.

S.int Inc.time	E	S1 1 day	S2 2 days	S3 3 days	S4 4 days
C	1	7.5	11.25	16.75	20.75
	2	3.75	7.25	9.5	
		3.0	7.0	9.25	
		3.0			
	3	3.97	6.56	7.7	11.16
	4	3.63	9.4	12.11	12.95
		2.84	8.61	10.84	12.14
		3.76	7.39	10.34	11.87
		2.34	8.41	10.04	11.47
	5	1.99			
		2.59			
		1.99			
	7	2.89	3.27	5.76	6.93
		4.15	5.93	8.7	9.35
		3.34	5.01	7.36	8.91
	8a	0.83	1.89	4.58	5.76
		1.18	3.05	4.61	5.36
Nb	1	2.5	4.0	6.0	6.0
		2.25	3.5	4.5	4.5
		3.75	4.75	6.0	7.0
		3.0	3.0	5.0	6.0
		2.5	4.75	7.5	9.0
		3.0	3.5	6.5	7.5
		2.75			
	2	2.0	4.25	6.5	11.75
		2.25	5.75	8.75	10.0
		2.5	4.25	7.75	10.0
		2.0	4.5	7.0	
	3	1.24	2.18	2.75	
		0.94	1.98	3.12	
		1.24	1.99	2.56	

TABLE 89 continued

	4	1.21	2.96	1.37	2.67
		Ø.Ø4	Ø.75	2.65	3.72
		1.1	1.81	5.61	6.72
		3.76	4.47	3.56	4.4
		Ø.71	3.Ø5	2.76	3.94
		Ø.71	1.92		
	5	1.99			
		1.76			
		2.23			
		2.23			
	6	1.28	2.86	5.75	7.44
		Ø.85	1.99	4.61	6.68
		1.43	2.86	5.75	8.5
		Ø.85	2.43	5.88	7.32
	7	1.48	2.52	3.92	4.32
		Ø.95	1.89	3.29	3.97
	8a	Ø.62	1.19	1.54	1.57
		Ø.62	1.38	1.98	2.Ø2
		Ø.62	1.Ø8	1.42	1.46
		Ø.35	1.Ø3	1.39	1.86
	4	Ø.29	2.63	4.Ø4	4.88
		Ø.29	2.63	3.58	4.5
		Ø.71	1.42	2.26	3.18
		1.75	4.1	5.16	7.31
	5	1.99			
Nb**					
	6	Ø.72	1.89	4.25	5.69
		Ø.45	2.95	5.81	8.18
		Ø.99	1.99	4.35	6.56
	7	Ø.79	1.94	3.88	4.16
	8a	Ø.44	Ø.78	1.27	1.49
		Ø.35	1.15	1.26	1.48
		Ø.28	Ø.62	1.11	1.33
		Ø.35	1.15	1.26	1.36

KEY : S.int = Sampling interval
Inc.time = Incubation time
E = Experiment number
Nb = N.brasiliensis-activated macrophages
Nb** = N.brasiliensis-activated macrophages + Ø.ØØ2% antigen in vitro

TABLE 90

7 day N.brasiliensis infection : specific activity of cumulative lysozyme released.

MØ Type	E	F	Cumulative lysozyme released (µg)	Specific activity (µg per mg of cell protein)
C	4	S6	15.09	105.17
			15.28	124.29
			14.78	85.2
			13.57	71.83
	7	S6	11.38	69.57
			12.60	77.33
			11.77	74.5
	8a	S4	5.76	21.94
			5.36	25.07
Nb	4	S6	3.92	19.52
			4.88	14.08
			8.64	35.52
			5.92	21.12
	6	S6	10.15	96.26
			8.28	52.9
			11.43	57.0
			9.77	59.2
	7	S6	6.20	44.22
			4.65	33.47
			5.09	25.62
	8a	S4	1.57	8.22
			2.02	13.2
			1.46	8.36
			1.86	10.0
Nb + 0.002% Ag in vitro	6	S6	9.07	66.9
			11.57	84.4
	8a	S4	1.49	8.76
			1.48	6.71
			1.33	7.07
			1.36	9.67

KEY: E = Experiment number
F = Final sample

TABLE 91

7 day N.brasiliensis infection : Lysozyme production over 3 days in culture : % secretion (Lysozyme expressed as µg lysozyme per 2 x 10^6 macrophages). Experimental series "B".

	E	S1 a	S1 b	S1 c	S1 d	S2 a	S2 b	S2 c	S2 d	S3 a	S3 b	S3 c	S3 d
C	8b	1.87	Ø.51	2.38	78.7	2.24	Ø.72	2.95	75.77	4.35	Ø.28	4.63	93.9
		1.31	Ø.21	1.52	86.4								
		1.31	Ø.51	1.82	72.16	2.63	Ø.61	3.24	81.3	4.88	Ø.20	5.Ø8	96.15
Nb	8b	Ø.44	Ø.Ø9	Ø.53	83.1	Ø.9	Ø.Ø3	Ø.93	96.8	1.85	Ø.13	1.98	93.6
		Ø.62	Ø.Ø4	Ø.66	94.5	Ø.8Ø	Ø.2Ø	1.Ø6	8Ø.41	2.Ø9	Ø.Ø7	2.16	96.9
		Ø.44	Ø.13	Ø.57	77.6	Ø.8Ø	Ø.Ø2	Ø.82	97.25	Ø.93	Ø.Ø4	Ø.97	96.4
		Ø.44	Ø.11	Ø.55	79.3					Ø.92	Ø.Ø8	1.Ø1	92.2
Nb**	8b	Ø.53	Ø.Ø6	Ø.58	89.5	Ø.92	Ø.Ø5	Ø.97	94.98	1.26	Ø.33	1.59	79.4
		Ø.44	Ø.Ø5	Ø.49	88.97	1.42	Ø.Ø8	1.5Ø	94.9	1.Ø8	Ø.38	1.46	73.9
		Ø.44	Ø.Ø7	Ø.51	85.7	1.15	Ø.Ø8	1.23	93.78	1.66	Ø.28	1.94	85.6
		Ø.44	Ø.Ø6	Ø.49	88.8	1.51	Ø.Ø9	1.61	93.95				

KEY: a = extracellular lysozyme; b = intracellular lysozyme; c = total lysozyme ; d = % secreted
Nb** = N.brasiliensis-activated macrophages + Ø.ØØ2% antigen in vitro

TABLE 92

7 day N.brasiliensis infection : lysozyme production by macrophages over 3 days in culture (µg lysozyme per mg of cell protein)

	E	S1 a	S1 b	S1 c	S2 a	S2 b	S2 c	S3 a	S3 b	S3 c
C	8b	7.91	2.14	10.05	8.10	2.59	10.68	15.11	0.98	16.09
		6.33	0.99	7.32	9.33	1.15	10.48	20.55	0.82	21.36
		5.58	2.15	7.73						
Nb	8b	2.60	0.53	3.14	3.13	0.10	3.23	11.91	0.81	12.72
		3.27	0.19	3.46	3.95	0.78	4.73	8.12	0.26	8.37
		2.45	0.71	3.16	3.57	0.1	3.67	3.86	0.15	4.01
		2.32	0.60	2.92				4.96	0.42	5.38
Nb**	8b	2.67	0.31	2.98	5.38	0.28	5.66	6.18	1.61	7.79
		2.36	0.29	2.66	6.14	0.33	6.46	5.14	1.81	6.95
		2.54	0.42	2.96	5.75	0.38	6.13	10.33	1.74	12.07
		2.08	0.26	2.34	6.46	0.42	6.87			

KEY: a = extracellular lysozyme; b = intracellular lysozyme; c = total lysozyme
E = Experiment no.
Na** = N.asteroides-activated macrophages + 0.002% antigen in vitro

TABLE 93

13 day N.asteroides infection : accumulation of lysozyme in conditioned medium over 4 days in culture. Experimental series "A". Data from 4 replicate experiments in 2 of which N.asteroides -activated macrophages were exposed in vitro to antigen. (µg of lysozyme/2 x 10^6 macrophages)

S.int Inc. time	E	S1 1 day	S2 2 days	S3 3 days	S4 4 days
C	1	0.67	3.61	7.04	10.11
		1.94	4.88	7.3	10.37
		0.67	3.09	6.03	9.1
	2	1.87	2.5	2.04	4.0
		0.45	1.39		
	3a	1.28	1.86	2.15	2.44
		2.19			
	4a	1.21	2.5	4.25	5.01
		1.05	3.23	4.27	5.36
Na	1	1.5	4.93	5.95	7.61
		1.5	4.19	4.63	6.29
		0.67	3.61	5.21	6.87
		1.5	4.19	5.21	
	2	0.71	2.19	3.16	6.61
		3.63	5.99	7.31	8.26
	3a	0.72	2.3	2.78	3.96
		0.72	1.3	1.68	3.07
		0.72	1.71	2.39	3.37
	4a	0.59	0.77	1.81	2.45
		0.59	1.5	2.02	2.5
Na + 0.002% Antigen in vitro	3a	0.85	3.04	3.75	5.83
	4a	0.29	0.94	1.84	2.07

KEY: S.int = Sampling interval
Inc.time = Incubation time
E = Experiment no.

TABLE 94

13 day N.asteroides infection : specific activity of cumulative lysozyme released.

MØ Type	E	F	Cumulative lysozyme released (µg)	Specific activity (µg per mg of cell protein)
C	2	S6	6.60	32.9
	4a	S4	5.01	26.98
			5.36	23.84
Na	2	S6	9.50	52.1
	4a	S4	2.45	28.8
			2.5	13.01
Na + Antigen in vitro	4a	S6	2.07	17.57

KEY: E = Experiment number
F = Final sample

TABLE 95

13 day N.asteroides infection : Lysozyme production over 3 days in culture : % secretion (Lysozyme expressed as µg lysozyme per 2 x 10^6 macrophages). Experimental series "B"

	E	S1 a	b	c	d	S2 a	b	c	d	S3 a	b	c	d
C	4b	1.21	0.28	1.49	81.39	2.66	0.12	2.78	95.9	3.81	0.15	3.96	96.2
		0.59	0.56	1.15	51.5	3.25	0.13	3.38	96.1	3.83	0.20	4.03	95.1
Na	4b	1.05	0.23	1.28	81.8	1.12	0.06	1.18	94.9	2.58	0.12	2.7	95.5
		0.59	0.19	0.78	75.7	1.22	0.05	1.27	95.7	1.56	0.11	1.67	93.5
Na**	4b	0.44	0.16	0.60	73.5	1.09	0.43	1.52	71.6	2.14	0.07	2.21	97
		0.29	0.18	0.47	61.4	1.04	0.14	1.18	87.8	1.38	0.08	1.46	94.5

KEY: a = extracellular lysozyme; b = intracellular lysozyme; c = total lysozyme; d = % secreted
E = Experiment no.
Na** = N.asteroides-activated macrophages + 0.002% antigen in vitro

TABLE 96

13 day N.asteroides infection : lysozyme production by macrophages over 3 days in culture (µg lysozyme per mg of cell protein)

	E	S1 a	b	c	S2 a	b	c	S3 a	b	c
C	4b	6.46	1.48	7.93	10.82	0.47	11.28	23.61	0.93	24.54
		3.91	5.6	9.51	17.73	0.72	18.45	18.02	0.94	18.95
Na	4b	7.71	1.7	9.41	6.71	0.36	7.07	12.71	0.6	13.31
		3.42	1.09	4.51	8.28	0.37	8.65	7.08	0.48	7.49
Na**	4b	3.16	1.14	4.3	5.52	2.19	7.71	11.94	0.37	12.31
		1.34	1.37	2.71	4.78	0.66	5.44	10.55	0.61	11.16

KEY: a = extracellular lysozyme; b = intracellular lysozyme; c = total lysozyme
E = Experiment no.
Na** = N.asteroides-activated macrophages + 0.002% antigen in vitro

TABLE 97

13 day N.brasiliensis infection : accumulation of lysozyme in conditioned medium over 4 days in culture. Experimental series "A". Data from 8 replicate experiments in 2 of which N.brasiliensis -activated macrophages were exposed in vitro to antigen. (µg of lysozyme/2 x 10^6 macrophages)

S.int Inc. time	E	S1 1 day	S2 2 days	S3 3 days	S4 4 days
C	1	0.67	3.61	7.04	10.11
		1.94	4.88	7.3	10.37
		0.6	3.09	6.03	9.1
	2	4.34	8.68	13.02	15.89
		3.35	7.19	12.04	14.91
	3	4.51	8.75	15.75	16.25
	4	2.51	5.5	9.75	13.0
	6	0.82	1.75	3.13	3.95
	7	1.86	4.07	5.5	7.08
		1.86	4.9	5.99	
	8a	1.99	4.76	6.16	8.55
		1.83	3.87	4.96	7.35
		1.83	3.85	5.09	6.98
		2.3	5.23	6.32	8.88
Nb	1	2.4	5.09	6.11	7.77
		1.5	3.64	4.66	6.15
		1.72	4.66	5.38	
	3	1.5	3.0	6.5	8.0
		0.75	1.5	5.0	7.75
		1.25	3.75	6.5	8.5
	4	3.5	6.0	10.0	14.75
		3.5	7.0	9.5	10.75
	5	0.62	1.44	1.78	
		0.82	1.44	1.78	
	7	0.95	2.27	3.04	4.32
		0.95	2.76	3.53	5.13
		1.2	3.27	3.74	5.02
	8a	1.52	2.97	3.72	5.28
		1.52	2.97	3.61	5.01
		1.52	2.42	2.92	4.01
Nb**	7	1.38	3.07	3.68	4.01
		1.29	2.85	3.78	
		0.94			
	8a	1.83	1.83	2.92	4.97
		1.52	2.97	3.46	4.70
		2.15	3.46	4.24	5.64

KEY: S.int = Sampling interval
Inc.time = Incubation time
Nb** = N.brasiliensis-activated macrophage cultures + 0.002% antigen in vitro

TABLE 98

13 day N.brasiliensis infection : specific activity of cumulative lysozyme released.

MØ Type	E	F	Cumulative lysozyme released (µg)	Specific activity (µg per mg of cell protein)
C	3	S1Ø	33.25	232.8
	7	S6	9.41	52.8
	8a	S4	8.55	43.69
			7.35	40.42
			6.98	23.51
			8.88	40.22
Nb	3	S1Ø	17.75	166.2
			20.75	103.75
			17.25	171.47
	7	S6	7.34	41.88
			8.85	30.33
			9.57	44.8
	8a	S4	5.28	24.2
			5.01	23.89
			4.01	23.14
Nb + 0.002% antigen in vitro	8a	S4	4.97	17.19
			4.70	27.27
			5.64	21.5

KEY: E = Experiment number
F = Final sample

TABLE 99

13 day N.brasiliensis infection : Lysozyme production over 3 days in culture : % secretion (Lysozyme expressed as µg lysozyme per 2 x 10^6 macrophages). Experimental series "B".

	E	S1 a	S1 b	S1 c	S1 d	S2 a	S2 b	S2 c	S2 d	S3 a	S3 b	S3 c	S3 d
C	8b	1.99	0.29	2.28	87.5	4.32	0.22	4.54	95.18	6.03	0.20	6.23	96.8
		2.15	0.32	2.47	87.1	3.88	0.19	4.07	95.29	5.39	0.27	5.66	95.3
		1.52	0.59	2.11	71.9	4.15	0.18	4.33	95.14	6.63	0.34	6.97	95.18
		2.15	0.78	2.93	73.5	4.01	0.21	4.22	95.11				
Nb	8b	1.83	0.27	2.09	87.5	2.69	0.04	2.73	98.58	3.49	0.70	4.19	83.3
		1.68	0.25	1.93	87.07	3.25	0.10	3.35	97.06	3.77	0.08	3.85	97.8
		1.52	0.59	2.11	71.9	2.72	0.19	2.91	93.36	4.06	0.08	4.14	98.1
Nb**	8b	1.52	0.40	2.17	69.99	3.3	0.17	3.47	95.14	3.33	0.08	3.41	97.7
		1.52	0.16	1.68	90.3	2.38	0.19	2.57	92.55	4.60	0.07	4.68	98.44
						1.00	0.02	1.02	97.6				

KEY: a = extracellular lysozyme; b = intracellular lysozyme; c = total lysozyme; d = % secreted
E = Experiment no.
Nb** = N.brasiliensis-activated macrophages +0.002% antigen in vitro

TABLE 100

13 day N.brasiliensis infection : lysozyme production by macrophages over 3 days in culture (µg lysozyme per mg of cell protein)

	E	S1 a	S1 b	S1 c	S2 a	S2 b	S2 c	S3 a	S3 b	S3 c
C	8b	4.78	0.69	5.47	16.13	0.81	16.95	20.14	0.66	20.80
		5.80	0.86	6.66	11.33	0.55	11.89	18.87	0.93	19.80
		2.59	1.01	3.6	12.81	0.54	13.35	21.64	1.10	22.74
		5.58	2.01	7.59	12.91	0.66	13.57			
Nb		7.62	1.09	8.71	12.56	0.18	12.74	17.43	3.5	20.93
		7.64	1.13	8.77	13.81	0.42	14.23	19.25	0.43	19.68
		7.68	3.0	10.68	10.06	0.72	10.78	25.1	0.48	25.59
Nb**		5.46	1.44	6.90	11.14	0.57	11.71	15.25	0.37	15.62
		5.93	0.64	6.56	11.95	0.96	12.91	21.72	0.39	22.11
					7.38	0.18	7.55			

KEY: a = extracellular lysozyme; b = intracellular lysozyme; c = total lysozyme
E = Experiment no.
Na** = N.brasiliensis-activated macrophages + 0.002% antigen in vitro

TABLE 101

21 day N.asteroides infection : Accumulation of lysozyme in conditioned medium over 4 days in culture. Experimental series "A". Data from 4 replicate experiments in 2 of which N.asteroides -activated macrophages were exposed in vitro to antigen. (µg of lysozyme/2 x 10^6 macrophages)

S.int Inc. time	E	S1 1 day	S2 2 days	S3 3 days	S4 4 days
C	1	3.35	9.24	12.11	
	2	0.67	5.52	7.46	10.1
	3a	2.13	4.41	5.06	5.55
	4a	2.46	4.37	5.92	8.67
		2.11	4.02	5.49	8.63
		1.19	2.95	4.07	6.63
Na	1	2.87	4.37	5.24	
	2	0.67	3.54	5.48	
	3a	1.08	1.87	2.66	4.83
	4a	0.44	1.79	2.52	3.81
		0.16	0.88	1.48	2.66
		0.25	0.47	1.11	2.66
		0.1	0.72	1.14	3.05
Na+ 0.002% Antigen in vitro	3a	0.65	1.59	2.38	3.56
	4a	0.44	0.75	1.25	2.05
		0.35	0.65	1.15	1.64
		0.1	0.22	0.71	2
		0.4	0.71	1.06	2.7
		0.62	1.03	1.31	2.35

KEY: S.int = Sampling interval
Inc.time = Incubation time
E = Experiment no.

TABLE 102

21 day N.asteroides infection : specific activity of cumulative lysozyme released.

MØ Type	E	F	Cumulative lysozyme released (µg)	Specific activity (µg per mg of cell protein)
C	3a	S4	5.55	39.32
	4a	S4	8.67	37.06
			8.63	35.57
			6.63	31.62
Na	3a	S4	4.83	63.61
	4a	S4	3.81	21.83
			2.66	20.31
			2.66	16.83
			3.05	19.15
Na + 0.002% Antigen in vitro	3a	S4	3.56	25.64
	4a	S4	2.05	16.5
			1.64	13.38
			2.0	18.76
			2.7	20.44
			2.35	19.64

KEY: E = Experiment number
F = Final sample

TABLE 103

21 day N.asteroides infection : Lysozyme production over 3 days in culture : % secretion (µg lysozyme/2 x 10^6 macrophages). Experimental series "B".

	E	S1 a	S1 b	S1 c	S1 d	S2 a	S2 b	S2 c	S2 d	S3 a	S3 b	S3 c	S3 d
C	3b	1.72	0.28	2.00	86.22	4.54	0.14	4.86	96.6	4.79	0.28	5.07	94.6
		2.9	0.39	3.29	88.26	4.42	0.02	4.44	99.6				
	4b	1.75	0.48	2.23	78.36	6.15	0.59	6.74	91.25	6.7	0.22	6.92	96.88
		1.75	0.23	1.98	88.3	5.8	0.56	6.36	91.26	6.59	1.19	7.78	84.73
		1.3	0.44	1.73	74.8	3.37	0.26	3.63	92.96	6.66	0.52	7.18	92.78
Na	3b	0.65	0.12	0.76	85.58	1.87	0.52	2.39	78.2	1.97	0.13	2.09	93.6
		0.37	0.13	0.51	73.94	1.88	0.03	1.91	98.7	1.96	0.03	1.99	98.4
		0.37	0.08	0.45	82.85	1.59	0.0011	1.59	99.9				
	4b	0.16	0.02	0.18	87.36	1.08	0.11	1.19	90.7	1.78	0.08	1.86	95.48
		0.44	0.09	0.53	83.5	0.48	0			0.96	0.09	1.04	91.6
		0.07	0.04	0.12	63.0	0.65	0.07	0.73	90.0	1.48	0.05	1.53	94.8
		0.15	0.02	0.18	90.0	0.22	0.06	0.28	78.4	0.82	0.07	0.89	92.4
Na**	3b	0.51	0.03	0.54	94.8	1.17	0.40	1.57	74.4	1.82	0.04	1.85	98.1
	4b	0.4	0.09	0.49	82.15	0.6	0			1.47	0.04	1.52	96.9
		0.4	0.08	0.48	84.1	0.30	0.07	0.38	80.6	2.27	0.10	2.37	95.0
		0.44	0.04	0.44	91.1	0.50	0			1.02	0.27	1.30	78.9
		0.29	0.0081	0.3	97.3	1.01	0.06	1.07	94.48	1.79	0.20	1.98	90.0

KEY: a = extracellular lysozyme; b = intracellular lysozyme; c = total lysozyme; d = % secreted
E = Experiment no.
Na** = N.asteroides-activated macrophages + 0.002% in vitro

TABLE 104

21 day N.asteroides infection : specific activity of lysozyme produced by macrophages over 3 days in culture. (Lysozyme expressed as µg of lysozyme/mg of cell protein)

		S1			S2			S3		
	E	a	b	c	a	b	c	a	b	c
C	3b	6.65	Ø.89	7.54	11.38	Ø.36	11.74	22.87	1.32	24.19
		4.14	Ø.66	4.8Ø	18.63	Ø.07	18.67			
	4b	5.51	1.52	6.84	18.87	2.6Ø	21.47	3Ø.Ø6	Ø.97	31.Ø5
		5.11	Ø.68	5.79	14.5	1.39	15.89	13.31	2.4Ø	15.71
		4.25	1.4	5.65	12.72	Ø.96	13.68	2Ø.62	1.61	22.22
Na	3b	2.26	Ø.38	2.64	7.28	2.Ø3	9.31	8.66	Ø.56	9.22
		1.17	Ø.41	1.58	6.46	Ø.Ø8	6.55	8.44	Ø.14	8.58
		1.31	Ø.27	1.58	6.Ø7	Ø.ØØ43	6.Ø7			
	4b	Ø.72	Ø.Ø8	Ø.81	5.46	Ø.56	6.Ø2	7.7Ø	Ø.37	8.Ø7
		1.64	Ø.32	1.96	2.37	Ø		2.65	Ø.24	2.89
		Ø.26	Ø.15	Ø.41	2.7Ø	Ø.3	3.ØØ	1Ø.17	Ø.35	1Ø.54
		Ø.58	Ø.Ø6	Ø.64	1.Ø5	Ø.29	1.34	4.84	Ø.4	5.24
Na**										
	3b	2.34	Ø.12	2.46	3.63	1.25	4.88	1Ø.53	Ø.2	1Ø.73
	4b	1.57	Ø.34	1.94	2.93	Ø		8.32	Ø.26	8.58
		1.72	Ø.32	2.Ø4	1.38	Ø.33	1.71	2Ø.34	Ø.88	21.22
		1.81	Ø.18	1.99	2.67	Ø		6.Ø3	1.61	7.64
		1.29	Ø.Ø4	1.32	5.Ø1	Ø.29	5.31	12.75	1.4	14.15

KEY: E = Experiment number
a = Extracellular lysozyme
b = Intracellular lysozyme
c = Total lysozyme
C = Control macrophages
Na = N.asteroides-activated macrophages
Na** = N.asteroides-activated macrophages + Ø.ØØ2% antigen in vitro

TABLE 105

21 day N.brasiliensis infection : Accumulation of lysozyme in conditioned medium over 4 days in culture. Experimental series "A". Data from 8 replicate experiments, in 1 of which N.brasiliensis -activated macrophages were exposed to antigen in vitro. Lysozyme expressed as µg of lysozyme per 2 x 10^6 macrophages.

S.int / Inc.time	E	S1 1 day	S2 2 days	S3 3 days	S4 4 days
C	1	0.75	1.0	3.0	5.25
	2	4.5	10.0	17.0	3.75
		2.0	10.0	18.0	24.5
	3	2.0	5.75	10.75	15.25
		2.0	4.0	9.75	13.75
	4	4.75	9.25	14.0	16.0
		4.0	7.5	10.5	12.5
	5	0.57	1.71	2.65	3.59
		0.57	1.13	2.08	
		1.14			
	6	4.21	9.22	10.91	13.35
		3.97	10.45	12.79	14.35
		3.14	8.31	9.63	10.78
	7	3.18	6.05	8.95	11.16
		2.01	5.15	8.19	10.26
		3.18	5.51	8.98	10.79
		2.9	7.45	10.2	12.54
		4.38	7.03	11.1	12.66
	8a	1.28	3.36	5.73	24.03
		1.16	3.65	5.47	12.5
		1.16	4.07	5.89	9.93
Nb	1	1.0	3.0	4.25	5.75
	2	4.75	10.75	16.75	23.75
	3	1.0	4.75	7.75	
		2.0	3.5		
	4	2.0	5.75	10.0	13.25
		2.0	5.25	9.0	14.0
	5	1.24	3.59		
		0.75	1.59		
	6	1.89	7.38	9.45	11.34
	7	2.21	4.97	9.35	12.1
	8a	2.1	4.46	5.9	8.66
		1.21	2.87	3.71	6.47
		1.21	3.1	4.23	6.47
Nb**	8a	2.1	3.76	4.75	8.75
		0.62	2.05	3.49	5.57

KEY : S.int = Sampling interval
Inc.time = Incubation time
E = Experiment number
C = Control macrophages
Nb = N.brasiliensis-activated macrophages
Nb** = N.brasiliensis-activated macrophage cultures + 0.002% antigen in vitro

TABLE 106

21 day *N.brasiliensis* infection : specific activity of cumulative lysozyme released.

	E	F	Cumulative lysozyme released (µg)	Specific activity (µg per mg of cell protein)
C	2	S6	34.25	185.69
			32.25	146.7
	4	S6	20.25	89.2
			16.0	48.0
	6	S6	17.49	107.96
			18.91	120.0
			13.86	125.2
	7	S6	16.72	128.22
			16.9	88.08
			18.04	153.59
			18.37	123.16
			22.38	200.21
	8a	S4	24.03	113.91
			12.5	59.96
			9.93	38.1
Nb	2	S6	30.5	227.1
	4	S6	19.25	66.6
			19.5	61.7
	6	S6	15.52	104.5
	7	S6	20.02	119.56
	8a	S4	8.66	18.08
			6.47	14.45
			6.47	19.62
Nb + 0.002% Ag *in vitro*	8a	S4	8.75	18.33
			5.57	16.21

KEY: E = Experiment number F = Final sample

TABLE 107

21 day N.brasiliensis infection : Lysozyme production over 3 days in culture : % secretion (Lysozyme expressed as µg lysozyme per 2 x 10^6 macrophages). Experimental series "B"

	E	S1 a	S1 b	S1 c	S1 d	S2 a	S2 b	S2 c	S2 d	S3 a	S3 b	S3 c	S3 d
C	8b	1.65	Ø.3Ø	1.95	84.55	3.51	Ø.62	4.13	85.1	5.37	Ø.Ø9	5.46	98.4
		1.28	Ø.31	1.59	8Ø.3	4.5	Ø.97	5.47	82.2	6.11	Ø.87	6.98	87.5
										5.99	Ø.32	6.31	94.9
Nb	8b	Ø.8	Ø.16	Ø.96	83.1	2.66	Ø.84	3.5Ø	76	4.86	Ø.64	5.5Ø	88.4
		Ø.61	Ø.Ø9	Ø.69	87.6	3.36	Ø.53	3.89	86.3	3.8	Ø.44	4.24	89.7
						2.24	Ø.6Ø	2.84	78.8	3.68	Ø.76	4.45	82.8
Nb**	8b	1.64	Ø.5Ø	2.14	76.5	2.87	Ø.38	3.25	88.2	3.51	Ø.59	4.1Ø	85.6
						2.5	Ø.34	2.84	87.98	2.75	Ø.7Ø	3.45	79.7

KEY: a = extracellular lysozyme; b = intracellular lysozyme; c = total lysozyme; d = % secreted
E = Experiment no.
Nb** = N.brasiliensis-activated macrophages + Ø.ØØ2% antigen in vitro

TABLE 108

21 day *N.brasiliensis* infection : lysozyme production by macrophages over 3 days in culture (µg lysozyme per mg of cell protein)

	E	S1 a	S1 b	S1 c	S2 a	S2 b	S2 c	S3 a	S3 b	S3 c
C	8b	5.56	1.02	6.58	13.76	2.42	15.91	31.60	0.52	32.12
		4.81	1.12	5.99	16.08	3.48	19.55	24.13	3.44	27.57
								27.82	1.50	29.32
Nb	8b	2.18	0.44	2.62	6.77	2.14	8.10	9.66	1.27	10.93
		1.40	0.19	1.59	9.7	1.54	11.24	10.04	1.15	11.19
					6.71	1.81	8.52	11.33	2.35	13.68
Nb**	8b				6.66	0.89	7.55	9.36	1.58	10.94
		3.66	1.13	4.79	7.3	1.00	8.30	6.77	1.72	8.49

KEY: a = extracellular lysozyme; b = intracellular lysozyme; c = total lysozyme
E = Experiment no.
Nb** = *N.brasiliensis*-activated macrophages + 0.002% antigen *in vitro*

TABLE 109

2 day N.asteroides infection. Median lysozyme and their 95% confidence limits for N.asteroides-activated macrophages at each sampling interval. Median lysozyme values expressed as percentages of their own controls.

Sampling Interval		S1	S2	S3	S4
C	Median	1.90	3.17	6.27	9.25
	LL	1.28	2.08	4.01	5.84
	UL	2.56	8.36	8.53	12.66
Na	Median	1.52	3.11	4.86	5.9
	LL	1.12	2.49	3.88	4.93
	UL	2.05	3.67	5.52	6.7
	%C	80	98.1	77.5	63.8

TABLE 110

2 day N.brasiliensis infection : Median lysozyme values expressed as percentage controls.

Sampling Interval		S1	S2	S3	S4
C	Median	2.47	4.34	6.45	8.64
	LL	1.78	3.4	4.72	5.71
	UL	3.85	9.17	14.04	16.15
Nb	Median	2.47	4.59	6.32	9.00
	LL	1.93	3.41	4.64	5.94
	UL	3.4	5.79	8.86	12.49
	% C	100	105.7	97.98	104.17
Nb+ 0.002% Ag	Median	3.45	5.35	6.94	8.56
	LL	2.22	4.15	4.97	5.5
	UL	5.3	9.03	12.85	15.15
	%C	139.68	123.27	107.6	99.07

TABLE 111

Seven day N.asteroides infection : median lysozyme values expressed as percentage controls

Sampling Interval		S1	S2	S3	S4
Control macrophage cultures	M	1.82	4.04	3.91	6.84
	LL	0.98	2.16	2.99	3.91
	UL	2.65	5.3	8.02	10.84
N.asteroides-activated macrophages	M	0.695	1.26	2.1	2.97
	LL	0.48	0.97	1.67	2.36
	UL	0.95	1.69	2.59	3.62
	%C	38.2	31.2	53.7	43.4
N.asteroides-activated macrophages + 0.002% Ag	M	0.6	1.13	1.998	3.42
	LL	0.36	0.72	1.27	1.98
	UL	1.26	2.06	2.43	3.71
	%C	32.97	27.97	51.1	50.0

TABLE 112

Seven day N.brasiliensis infection : median lysozyme values expressed as percentage controls

Sampling Interval		S1	S2	S3	S4
Control macrophage cultures	M	3.05	6.66	8.94	10.32
	LL	2.44	4.81	7.04	7.92
	UL	4.06	8.33	10.81	13.06
N.brasiliensis - activated macrophages	M	1.62	2.89	4.46	5.70
	LL	1.19	2.27	3.58	4.36
	UL	2.12	3.43	5.44	7.06
	%C	53.1	43.4	49.88	55.23
N.brasiliensis - activated macrophages + 0.002% Ag	M	0.58	1.89	3.18	4.24
	LL	0.37	1.28	1.77	2.34
	UL	1.14	2.63	4.35	5.91
	%C	19.01	28.38	35.57	41.09

TABLE 113

13 day N.asteroides infection : median lysozyme values expressed as percentage controls.

Sampling Interval		S1	S2	S3	S4
Control Macrophage Cultures	M	1.27	2.83	4.63	6.80
	UL	0.67	1.95	2.1	3.28
	UL	1.87	3.99	3.17	10.24
N.asteroides - Activated Macrophages	M	1.05	2.93	3.80	5.06
	LL	0.66	1.61	2.30	3.07
	UL	2.11	4.19	5.21	7.11
	%C	82.68	103.53	82.07	74.41

TABLE 114

Thirteen day N.brasiliensis infection : median lysozyme values expressed as percentage controls.

Sampling Interval		S1	S2	S3	S4
Control macrophage cultures	M	2.02	4.79	6.81	9.99
	LL	1.34	3.85	5.56	7.82
	UL	3.09	6.3	9.75	12.57
N.brasiliensis - activated macrophages	M	1.42	3.23	4.62	6.53
	LL	1.1	2.42	3.39	5.08
	UL	2.13	4.22	6.31	9.26
	%C	70.29	67.43	67.84	65.37
N.brasiliensis - activated macrophages + 0.002% Ag	M	1.52	2.96	3.62	4.83
	LL	1.28	2.55	3.29	4.25
	UL	1.76	3.37	3.95	5.41
	%C	75.25	61.8	53.16	48.35

TABLE 115

Twenty-one day *N.asteroides* infection : median lysozyme values expressed as percentage controls.

Sampling Interval		S1	S2	S3	S4
Control macrophage cultures	M	2.11	4.41	5.92	7.83
	UL	1.58	3.34	4.32	6.68
	UL	2.65	5.48	7.52	8.98
N.asteroides-activated macrophages	M	Ø.57	1.85	2.63	3.24
	LL	Ø.13	Ø.395	1.13	2.69
	UL	1.98	3.96	5.36	3.79
	%C	27.Ø1	41.95	44.43	41.38
N.asteroides-activated macrophages + Ø.ØØ2% Ag	M	Ø.42	Ø.75	1.2	2.35
	LL	Ø.31	Ø.48	Ø.87	2.69
	UL	Ø.53	1.Ø2	1.53	2.73
	%C	19.91	17.Ø	20.27	3Ø.Ø1

TABLE 116

Twenty-one day *N.brasiliensis* infection : median lysozyme values expressed as percentage controls.

Sampling Interval		S1	S2	S3	S4
Control macrophage cultures	M	2.59	6.Ø2	9.51	12.94
	LL	1.95	4.58	6.96	11.16
	UL	3.38	7.58	11.21	17.Ø1
N.brasiliensis-activated macrophages	M	1.61	4.38	7.63	1Ø.24
	LL	1.21	3.25	5.Ø7	6.47
	UL	2.11	6.17	1Ø.5	16.21
	%C	62.16	72.76	8Ø.23	79.13

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SECTION III

A PHASE CONTRAST AND SCANNING ELECTRON MICROSCOPY STUDY : INFLUENCE OF PREVIOUS INOCULATION WITH N.ASTEROIDES OR N.BRASILIENSIS ON MURINE MACROPHAGE MORPHOLOGY

CHAPTER 5

PART 1 : MORPHOLOGICAL CHARACTERISTICS OF MACROPHAGES FROM MICE PREVIOUSLY INFECTED WITH N.ASTEROIDES OR N.BRASILIENSIS : A STUDY USING THE PHASE-CONTRAST INVERTED MICROSCOPE.

PART 2 : A SCANNING ELECTRON MICROSCOPY STUDY OF THE SURFACE CHARACTERISTICS OF MACROPHAGES FROM MICE PREVIOUSLY INOCULATED WITH N.ASTEROIDES OR N.BRASILIENSIS

Chapter 5

PART 1 : MORPHOLOGICAL CHARACTERISTICS OF MACROPHAGES FROM MICE PREVIOUSLY INFECTED WITH N.ASTEROIDES OR N.BRASILIENSIS : A STUDY USING THE PHASE-CONTRAST INVERTED MICROSCOPE.

5.1 INTRODUCTION

In addition to the numerous biochemical and functional manifestations of a state of activation, macrophages harvested from animals recently infected with any of a variety of facultative intracellular parasites show a number of characteristic morphological changes when compared to normal cells (Blanden, 1968; Mackaness, 1970 : 62; Lazdins et al, 1978; Johnston, 1982).

5.1.1 MORPHOLOGICAL FEATURES OF ACTIVATED MACROPHAGES : PHASE-CONTRAST MICROSCOPY

The morphology of the macrophage varies depending on its state of activity (Elhelu, 1983). The mononuclear phagocytes in the normal mouse peritoneal cavity are young monocyte-like cells (Cohn and Benson, 1965a); the shape of the nucleus is reniform but may vary from horse-shoe shaped to fusiform (Fedorko and Hirsch, 1970; Lessin and Bessis, 1972 : 736); they attach firmly and subsequently spread on plastic or glass surfaces in culture and become highly polarised and oriented as time in culture progresses. On phase contrast microscopy, secondary lysozomes (phase dense granules, Polliack and Gordon, 1975) and

endocytic vesicles are seen in the perinuclear area. As the cell spreads, pseudopods start to form, there is a ruffling of the membrane borders and mitochondria appear as phase-dense rods scattered through the cytoplasm. With more prolonged time in culture there is a progressive increase in the size of the cell and the number of phase-dense granules (Cohn and Benson, 1965; Cohn, 1968; Cohn, 1975).

Macrophage activation is manifested by a variety of morphological changes. The most characteristic features are their content of mitochondria (filamentous phase-dense bodies), lysozomes (spherical phase-dense granules) and pinocytic activity (number of phase- lucent vesicles). Increased numbers of lysozomes and levels of lysozomal enzymes has been accepted as one of the most conspicuous features during in vivo activation (Blanden, 1968; Mackaness, 1970 : 62; Reikvam, Grammeltdveldt and Hoiby, 1975). Typically, lysozomes and pinocytic vesicles are seen in the perinuclear area in activated macrophages (Nogueira and Cohn, 1978; Reikvam, Grammeltveldt and Hoiby, 1975).

Another morphological change which is very apparent in activated macrophages is its larger size, which is reflected in its increased protein content (Cohn, 1978). Activation is manifested by a lower nucleo-cytoplasmic ratio (McLaughlin, Ruddle and Waksman, 1972) ie cells from infected animals seem to have a much more extensive cytoplasm (Blanden, 1968) with large numbers of mitochondria. Noguiera and Cohn (1978) noted that activated macrophages spread symmetrically and had intensely

ruffled plasma membranes. Macrophages from BCG-infected animals spread more rapidly than control macrophages and assumed a rounded shape whereas the normal cells became elongated (Mackaness, 1970; : 62). Increased ruffling of the membrane and prominence of pseudopodia are also characterisic of macrophages that have been exposed to irritants (Ogmundsdottir and Weir, 1980). Lipid droplets are prominent in some stimulated macrophages (Hirsch and Fedorko, 1970 : 16) and increased adhesiveness is a well known property of activated macrophages (Hopper, Wood and Nelson, 1979; Lasser 1983).

5.1.2 CHARACTERISTICS OF ACTIVATED MACROPHAGES : MORPHOLOGY AS A PARAMETER

Morphological changes are characteristic of activated macrophages. Changes which are most commonly documented as being characteristic are increased adhesiveness, rate and extent of spreading, increased ruffling of the membrane and prominence of pseudopodia, increased number of cytoplasmic granules and vacuoles (Ogmundsdottir and Weir, 1980; Hopper, Wood and Nelson, 1979; Lasser, 1983). In addition, it has been suggested that the morphology of macrophages and their *in vitro* behaviour reliably portray their antimicrobial activity in vivo (Blanden, Lefford and Mackaness, 1969). Macrophage morphology was therefore selected as a parameter of macrophage activation.

5.2 METHOD

Macrophage cultures were monitored by phase contrast microscopy throughout the culture period. Monolayer cultures of macrophages

from mice previously inoculated with live Nocardia organisms were compared with cultures from saline-inoculated mice incubated under identical conditions and always run in parallel. Macrophage morphology was monitored in living monolayer cultures by phase-contrast microscopy using a Leitz Diavert Inverted Microscope (Wild Heerbrug, Switzerland) fitted with 10, 20 and 32x phase-contrast objectives and 16x eyepieces. The Leitz Diavert inverted microscope was fitted with a Wild MPS 61 camera with a 10x eyepiece and a Wild MPS 55 Photoautomat box and photographs were taken on Ilford PAN F black and white film.

5.3 RESULTS

Culture conditions, such as presence or absence of serum, were found to influence the morphology of macrophages (Cohn and Benson, 1965b), whether from Nocardia-inoculated or control mice. Therefore cultures were always run in parallel. Generally, normal macrophages show two basic types of morphology in culture: either they are extended or stellate or they are rounded with abundant cytoplasm. With increasing time in culture the proportion of stellate cells increases, in agreement with the findings of Stuart, Habeshaw and Davidson(1978 : 31.6).

Phase-contrast micrographs of macrophage cultures destined for assay of lysozyme and plasminogen activator release (direct and indirect method) and for scanning electron microscopy were taken. For details of culture conditions see 3.2.3, 3.2.4, 4.2.1 and 2.8. The morphology of Nocardia-activated macrophages was distinctly different from that of control macrophages

irrespective of culture conditions. Appearance of control cells was dependent on the culture medium and macrophages in general assumed a characteristic morphology in the same media in replicate experiments. This is also true for Nocardia-activated macrophages, but to a lesser extent.

5.3.1 TWO DAY N.ASTEROIDES INFECTION

Typical phase-contrast micrographs of living macrophages from mice inoculated 2 days previously with N.asteroides or normal saline only are shown in Figs 43, 44. Control cells are present predominantly in the rounded form (cells have been in culture for 13.5 hours). The N.asteroides-activated macrophages are larger and are more well-spread, contain more lysozomes and phase-lucent vesicles. Cells are rounded with abundant cytoplasm or are stellate or dendritic in shape.

5.3.2 TWO DAY N.BRASILIENSIS INFECTION

Two day N.brasiliensis-activated macrophages in general appear larger than controls, have longer pseudopodia, contain more phase dense granules and are more irregularly shaped than control cells, irrespective of culture conditions. Phase contrast micrographs are shown in Figs 44, 45 (13.5 hour incubation).
Macrophages harvested from mice inoculated 2 days previously with N.asteroides or N.brasiliensis and cultured for 13.5 hours exhibit increased size, increased and more rapid spreading, increased pinocytic activity, ruffled membrane activity, increased numbers of perinuclear lysozomes, irregular shapes with long pseudopodia and frequently spread symmetrically, as shown in

Figs 43, 45.

Nocardia-activated macrophages showed more rapid spreading than control cells after 30 minutes of incubation, and the difference in rate of spreading was even more marked after 2.5 hours of incubation.

5.3.3 SEVEN DAY N.ASTEROIDES INFECTION

Macrophages from mice inoculated 7 days previously with N.asteroides appear to contain more phase dense granules and phase translucent vesicles than control cells. They also seem to be more well spread and to assume more irregular shapes and to have longer pseudopodia than controls. In some cultures (eg indirect PA) macrophages are large and predominantly rounded with extensive cytoplasm and numerous perinuclear lysozomes and vesicles, whereas controls are generally stellate, have fewer granules and are not as large. Cells in culture for direct assay for PA are shown in Fig 46. In this culture medium, the Nocardia-activated macrophages have assumed a stellate conformation and pseudopodia are prominent, as are perinuclear phase dense granules. Control cells (not shown) were similar in appearance to those shown in Fig 47.

Macrophages from mice inoculated 7 days previously with N.asteroides show more rapid spreading than controls after 2 hours of incubation.

5.3.4 SEVEN DAY N.BRASILIENSIS INFECTION

In the phase-contrast micrographs shown in Figs 47, 48 (3 day cultures) N.brasiliensis-activated macrophages are larger than control cells, they have assumed a rounded morphology with a more extensive cytoplasm and vast numbers of lysozomes in the perinuclear cytoplasm. Some of the N.brasiliensis-activated cells have assumed a stellate conformation with long pseudopodia. Other cultures of N.brasiliensis-activated macrophage were predominently stellate or dendritic in shape with very extensive pseudopodia, numerous perinuclear lysozomes and were larger than controls. Increased rate of spreading of N.brasilinsis-activated cells compared to control cells was noted after 2 hours in culture.

5.3.5 THIRTEEN DAY N.ASTEROIDES INFECTION

The morphology of thirteen day N.asteroides-activated macrophages was distinctly different from control macrophages, irrespective of culture conditions. Control macrophages in culture for indirect PA assay (serum-free medium) were very elongated or stellate in shape whereas the Nocardia-activated macrophages were more symmetrically spread, appeared larger than controls and contained numerous perinuclear lysozomes and pinocytic vesicles. These cells were either rounded with extensive cytoplasm or were stellate with numerous long pseudopodia. In Figs 49, 50 (40 hour incubation), macrophages in culture for direct PA assay are shown. In this culture, control cells contain numerous pinocytic vesicles whereas N.asteroides-activated macrophages

contain very few. The latter cells have numerous long pseudopodia and are stellate in shape or are rounded with extensive cytoplasm and prominent perinuclear lysozomes. In addition, these cells exhibited more rapid spreading after 2.5 hours in culture than control cells.

5.3.6 THIRTEEN DAY N.BRASILIENSIS INFECTION

Thirteen day N.brasiliensis-activated macrophages were distinctly different from control cells, as was found in the cell cultures described above. General characteristics of N.brasiliensis-activated macrophages are increased numbers of perinuclear lysozomes, increased pinocytic activity (increased numbers of phase-dense vesicles), cells appear to be larger and to have assumed a more extreme stellate conformation than controls. These cells also tended to have many long thin pseudopodia. As in the cultures described above, N.brasiliensis-activated macrophages were present as large rounded cells with extensive cytoplasm and numerous perinuclear lysozomes.

In Figs 51, 52 the cells have been cultured for 2.5 hours. N.brasiliensis cells appear to spread more rapidly and extensively than control cells, to assume more stellate or dendritic conformations, to have increased lysozomal activity and to have increased ruffled membrane activity, compared to control cells.

5.3.7 TWENTY-ONE DAY N.ASTEROIDES INFECTION

Characteristics of cultured macrophages from mice inoculated twenty-one days previously with N.asteroides and compared with controls were increased number of phase-dense granules (lysozomes) in the perinuclear area, increased pinocytic activity and cells were generally rounded and symmetrically spread with extensive cytoplasm or were stellate with numerous irregular pseudopodia. Macrophages in Figs 53, 54 were cultured for 24 hours. It is difficult to determine from these photomicrographs whether increased spreading has occured in the Nocardia-activated cultures, however, symmetrically spread cells with extensive cytoplasm are a feature of the N.asteroides -activated cell culture. More rapid spreading of Nocardia -activated macrophages than control cells was noted after 2.5 hours in culture.

5.3.8 TWENTY-ONE DAY N.BRASILIENSIS INFECTION

Morphology of twenty-one day N.brasiliensis-activated macrophages is similar to the morphology of macrophages harvested from mice at the various times post-inoculation described above, therefore photomicro-graphs are not shown. Characteristics are increased numbers of phase dense granules and tendency of cells to become markedly stellate with long pseudopodia or rounded with extensive cytoplasm, compared to the controls. Increased spreading was noted in N.brasiliensis-activated macrophage cultures compared to control cells after 2.5 hours in culture.

5.3.9 PROTEIN CONTENT AS AN EXPRESSION OF CELL SIZE

Activated macrophages exhibit increased size, which is reflected in their increased protein content (Cohn, 1978). However, the protein content per 10^6 cells from BCG-vaccinated mice dropped in comparison to control cells (Kondo and Kanai, 1977) and macrophages from BCG-treated mice had a lower protein content than those injected with thioglycolate medium (Nathan and Root, 1977). To evalulate whether the apparent increase in size of macrophages harvested from mice previously inoculated with Nocardia was associated with an increase in protein content, macrophages were harvested from mice 2 days post-inoculation, glass adhered and washed after 2 hours in culture, incubated for a further 10 hours and protein content determined spectrofluorimetrically as described previously (2.3). A typical experiment is shown in Table 117, and it appears from these data that protein content is similar in control, N.asteroides-activated and N.brasiliensis-activated macrophages.

5.4 DISCUSSION

Macrophages harvested from mice recently infected with N.asteroides and N.brasiliensis are activated according to the parameter of morphological change. Differences in morphology characteristic of activated macrophages were observed in both N.asteroides and N.brasiliensis-activated macrophages over the two to twenty-one day period post-inoculation under study. Differences in macrophage appearance between control and Nocardia

macrophage cultures were found in all experiments. The most characteristic changes were increased numbers of perinuclear lysozomes and changes in the shape of the macrophages in the cultures of cells from animals infected with Nocardia. In general Nocardia- activated macrophages appear to be larger than controls, although sometimes this difference was marginal. Difficulties in determining whether Nocardia-activated macrophages are larger than control cells was generally experienced only in older cultures. Unstimulated cells have been described as remaining rounded for several hours but after 24 hours in culture are well spread with active membrane ruffling. Thioglycolate or endotoxin stimulated cells spread rapidly within 30 minutes, and remained well spread during further cultivation but resembled unstimulated cells more closely after 2 days (Polliack and Gordon, 1975).

Macrophages from mice recently infected with intracellular parasites such as mycobacteria exhibit an increase in size and spreading (Johnston et al, 1982), and enhanced spreading of macrophages on glass has been defined as a property of activated macrophages (Johnston, Chadwick and Cohn, 1981). Macrophages from mice previously inoculated with Nocardia spread more rapidly and more extensively and certainly appeared larger than control cells early in culture (Figs 43, 44, 45, 51, 52). Morphology of macrophages from Nocardia-inoculated mice does not appear to be related to period of infection or to be different in N.asteroides and N.brasiliensis infections.

This study was performed on living cells in tissue culture. The failure to note distinct mitochondria and consistently enhanced pinocytic activity may perhaps be accounted for by the difficulties encountered in photography of cells in tissue culture. It would therefore be useful to perform a study of Wright-Giemsa stained macrophages after varying periods in culture.

Morland and Kaplan (1977) point out that although morphological data have been widely used as criteria for macrophage activation, their study showed that such criteria are ambiguous and difficult to evaluate. They consider that not only spreading and granular content should be taken into account, but also the extent of ruffling and details of cell surface morphology should be considered. These aspects are detailed in part 2 of this chapter. However, phase contrast morphology of living cells is a useful method of examining cells free of fixation artifacts, and many of the larger organelles may readily be seen under these conditions. From this study it is apparent that the morphological appearance of macrophages from mice recently infected with Nocardia differs from that of unstimulated cells and that several morphological criteria of macrophage activation are fulfilled by these cells.

PART II : A SCANNING ELECTRON MICROSCOPY STUDY OF THE SURFACE CHARACTERISTICS OF MACROPHAGES FROM MICE PREVIOUSLY INOCULATED WITH N.ASTEROIDES AND N.BRASILIENSIS.

5.6 INTRODUCTION

The development of the scanning electron microscope (SEM) has radically altered the visualization of cell surface morphology and has provided a new ultrastructural parameter which greatly adds to the information accumulated by more conventional approaches (Walters, Papadimitriou and Robertson, 1976) such as light microscopy and transmission electron microscopy. The great depth of field of the SEM allows visualisation of the overall three- dimensional surface morphology of individual cells, and of the fine structures present on the cell surface (Papadimitriou, Finlay-Jones and Walters, 1973). The technique also reveals details of the spatial relationships of cells in culture and of cell-to-cell interactions.

5.6.1 NOMENCLATURE OF SURFACE MORPHOLOGICAL FEATURES

Many terms are used to describe the surface projections of macrophages. Newell (1980 : 231) has stated that definition of surface features of leukocytes is extremely difficult since surface projections are transient in nature and their shape and size are greatly affected by changes in their environment and by preparation procedures.

There appears to be a variety of descriptions of the features of the macrophage cell surface in the literature (Finch et al, 1982;

Carr 1980 : 260; Newell, 1980 : 231; Oliver and Berlin, 1982; Saint-Guillain et al, 1981; Papadimitriou, Finlay-Jones and Walters, 1973). It was felt that it would be helpful to define the terms used to describe surface features in this study at this point. Criteria for the identification of surface features are given in Table 118. Varying combinations of these surface features distinguish the surface ultrastructure of the macrophage (Carr, 1980 : 260).

5.6.2 SURFACE STRUCTURE OF NORMAL MOUSE PERITONEAL MACROPHAGES

The first SEM description of macrophage surfaces by Carr, Clarke and Salisbury (1969) gave a new perspective of the function and morphology of macrophages and lead to a number of studies of macrophage surfaces, workers concentrating on improved specimen preparations and hence preservation of membrane processes (Warfel and Elberg, 1970; Albrecht et al, 1972; Polliack and Gordon, 1975).

Numerous cell shapes, ranging from compact and spherical to completely spread, elongated (polarized), irregularly shaped or stellate have been observed in macrophages (Finch et al 1982; Warfel and Elberg, 1970; Johnson and Davies, 1981). Typical morphology of spherical and spreading macrophages adherent on glass coverslips is described by Polliack and Gordon (1975), Albrecht, Jordan and Hong, (1978) and Johnson and Davies (1981). Spreading cells typically exhibit pseudopodia and together with spherical cells have ruffles and ridges which traverse the

surface in irregular patterns, microvilli and lamellipodia and sometimes blebs. Spreading cells attach to the glass by an extension of the membrane which appears as transparent veils of cytoplasm which spread centrifugally over the substrate and are smooth compared to the main cell body. The cell thus becomes flatter and smoother as spreading progresses, ruffling or rolling of the veil edges or generation of long thin thread-like processes (filopodia) follows (Warfel and Elberg, 1970; Newell, 1980 : 264, 265; Walters, Papadimitriou and Robertson, 1976). An increased flattening with time in culture was observed with great variation in shape and degree of spreading (Polliack and Gordon, 1975). Macrophages settled on glass have been described as having a "fried egg" appearance (Carr, 1980 : 261) - the smooth flat surface of the peripheral cytoplasmic veils becomes raised over the main body of the cell - the nucleus is centrally orientated - and this area has an extensive series of membranes of various shapes and sizes on its surface (Polliack and Gordon, 1975; Warfel and Elberg, 1970; Newell 1980 : 265). Elongated and stellate cell surfaces were also associated with microvilli or ridges but these features occurred far less frequently than with the spherical forms (Johnson and Davies, 1981). The range of different cell shapes seen in macrophage cultures suggests that retraction and spreading of cells occurs continuously (Newell, 1980 : 265).

The surface morphology of adherent cells does not reflect their morphology <u>in situ</u> (Newell, 1980 : 264); and the macrophage surface structure is influenced by the characteristics of the

substrate to which it is attatched (Daems 1980 : 62).

5.6.3 EFFECTS OF VARIOUS AGENTS ON MACROPHAGE MORPHOLOGY : SEM STUDIES

In their original study, Carr, Clarke and Salsbury (1969) found that cells stimulated with glycerol trioleate (i.p. injection) were more irregular in shape, had rougher surfaces and showed more prominent ridge-like and flange-like processes than controls. A number of studies followed this and the effect of a wide variety of agents on macrophage surface morphology has been documented. i.p. Injection of mineral oil gave rise to cells that were markedly polar with many ridges and villi and some ruffling at the ends of long pseudopods (Morland and Kaplan, 1977); paraffin-induced macrophages were larger and flatter than controls with a closely packed pattern of ridges or flaps and more numerous spherules (Papadimitriou, Finlay-Jones and Walters, 1973). Thioglycolate and endotoxin-elicited macrophages showed greatly enhanced membrane activity - extensive spreading, ie flatter cells, larger surface area with prominent ruffles, ridge-like profiles and more frequent filopodia, also multiple perforations and cytoplasmic pits compared to control cells (Polliack and Gordon, 1975). Morland and Kaplan (1977) found similar results with endotoxin. Daems (1980 : 96) makes the comment that the exudate macrophage population is much more pleomorphic than that in the unstimulated peritoneal cavity, since differentiation of monocytes into macrophages occurs during the development of the inflammatory process.

Guttner et al (1975) attempted to correlate macrophage functional status with morphology by using chemotherapeutic agents which reduce phagocytosis. These agents made cell surface projections thicker, shorter and fewer in number, whereas antimetabolites which did not reduce phagocytosis did not produce any cell surface changes. Rat peritoneal macrophages rounded up in the presence of zinc - treated cells were mostly spherical with only a few showing radical protrusions and extensive pseudopodia were rare (Chvapil et al, 1977). Guinea pig macrophages exposed in vitro to a benzoquinone (shown to provide complete nonspecific immunity against lethal pathogens in mice) were larger with more extensive ruffles and lamellipodia formation was more extensive and occurred more frequently - this data confirmed others' findings that activated phagocytes are associated with extensive plasma membrane ruffling, filopodia and lamellipodia (Biemesderfer et al, 1978; Polliack and Gordon, 1975).

5.6.3.1 MODIFICATION OF MACROPHAGE SURFACE STRUCTURES IN IN VIVO AND IN VITRO INFECTION : SEM AS A PARAMETER IN THIS STUDY

Morphological changes are known to accompany the transformation of a macrophage from a resting to an activated state, such as occurs in chronic intracellular infection (Morland and Kaplan, 1977). Other observers have found changes in plasma membrane surface architecture in resting and activated macrophages using SEM. Peritoneal macrophages from mice previously immunized with Trypanosoma Cruzi had the appearance of stimulated or activated macrophages with extensive spreading and numerous fine filopodia,

which were often branched (Reed, Douglas and Speer, 1982; Polliack and Gordon, 1975; Morland and Kaplan, 1977).

Virulent and less virulent strains of N.asteroides were added to cultures of rabbit alveolar macrophages and their interaction studied by SEM. Macrophage response appeared to depend on the strain of the Nocardia infecting them. The virulent strain induced a high degree of macrophage aggregation leading to the formation of giant cells and destruction of the intracellular Nocardia, whereas few giant cells were noted in cultures infected with the less virulent strain. In the presence of Nocardia, macrophages became rounded and the organisms were phagocytosed. Macrophages that had phagocytosed Nocardia no longer spread on the glass surface, but remained rounded. Macrophages containing large numbers of organisms became smooth surfaced spheres with the Nocardia filaments projecting through the cell cytoplasm (Beaman, 1977).

Exposure of macrophages to mycoplasmas in vitro in the presence of antimycoplasma antibody gave rise to cells with a diffusely granular appearance. Increased occurrence of ruffled membranes, large and small lacunae and folding over of the mycoplasmas was noted (Jones, Minick and Young, 1977).

This SEM study was done as an extension of the phase contrast microscopy studies described in part 1, since it is important to consider the fine details of surface morphology of cells when evaluating morphology as a parameter of macrophage activation

(Morland and Kaplan, 1977). In addition, SEM can be used for cell identification, since there are certain features by which the macrophage can be recognized (Polliack et al, 1974). However, some functional marker is required for confirmation (Carr, 1980 : 260). The combination of cell morphology with lysozyme release would therefore provide conclusive identification.

5.7 METHODS

Specimen preparative techniques are extremely important in the preservation of delicate cell surface structures, and there are several reviews of the procedures for cell preparation (Boyde, 1980; Bell and Revel, 1980).

The method used was a modification of the method of Polliack and Gordon (1975). Macrophages were cultured as described previously (Section 2.8). After 40 hours in culture, glass coverslips layered with peritoneal cells were examined by phase contrast microscopy and were washed twice with HBSS at 37°C and then fixed for 5 minutes with 2% glutaraldehyde (8% aqueous solution, TAAB Laboratories Equipment, Reading, Berks UK). Coverslips were then rinsed twice in HBSS at 37°C. Physiological temperature was used in order to minimise distortion. Fresh fixative was then layered over the coverslips for a further 10 minutes at 37°C. Samples were then placed at 4°C in 2% glutaraldehyde for at least 24 hours. Coverslips were then rinsed twice in sodium cacodylate buffer (0.1M, pH 7.2 with sodium hydroxide 0.2M) to remove any unreacted fixative and then

post-fixed in 2% Osmium tetroxide for 1 hour (O_sO_4, Merck) in S-collidine (Polysciences Inc, Warrington, Penna 18976; pH 7.2, prepared according to manufacturer's instructions). Cells are washed prior to fixation to remove serum proteins and cell-secreted substances which would otherwise be fixed onto cell surfaces (Bell and Revel, 1980 : 4). 2% Glutaraldehyde appears to give acceptable results with most cells and is the usual concentration used (Bell and Revel, 1980 : 5). Post-fixation with O_sO_4 is not an absolute requirement for SEM but it aids in prevention of the elution of membrane lipids during dehydration, which appear as small cell surface pits (Newell, 1980 : 225). The main consideration in SEM is to stabilise the cell surface characteristics sufficiently so that they are not altered by the harshness of subsequent processing (Bell and Revel; 1980 : 7). O_sO_4 fixation is recommended prior to critical point drying (CPD) in order to preserve fine details of membrane structure (Boyde, Bailey and Vesely, 1974).

Coverslips were washed three times with distilled water and dehydrated in a graded series of alcohol (5%, 10%, 20%, 40%, 60%, 70%, 80%, 90%, 100%) for 5 minutes each, with great care to avoid premature drying while transferring attatched cells from one solution to another - surface tension changes can induce alterations in surface morphology (Biemesderfer et al, 1978). Specimen shrinkage occurs during ethanol dehydration, the most rapid shrinkage occuring in 80% ethanol (Boyde, 1980). After dehydration, specimens were transferred to fresh absolute alcohol. The specimen was then transferred into the precooled

high pressure chamber of a Polaron Critical Point Dryer (CPD) apparatus and critical point drying was done using liquid CO_2 as the transition fluid. The CPD permits drying of a sample without exposure to surface tension and the distorting effects that accompany it (Bell and Revel, 1980 : 16).

Samples were then cut to fit Cambridge Series 100 stubs and mounted using conductive silver paint on the stub and coverslip edge. Stubs were mounted and sputter-coated under partial vacuum with Argon bleed. Samples were sputter coated with gold-palladium to a thickness of 25 nm in a modified Hummer 4. The sputter coater was used in pulsed mode to reduce heating effects.

Samples were then viewed in a Cambridge Stereoscan S180 scanning electron microscope at an accelerating voltage of 15 - 20 KV at a tilt angle of 35^{o}. Micrographs were recorded on Ilford FP4 film at instrument magnifications from x 300 - x 11 000. Size of macrophages was estimated by measuring cell diameters (Finch et al, 1982) and median diameter values and their 95% confidence limits calculated.

5.8 RESULTS

As in the study of cell morphology using phase contrast microscopy, control and Nocardia-activated macrophage cultures were always run in parallel. Low power scanning electron micrographs (about x 300) invariably revealed different morphological characteristics for control and Nocardia-activated

cells when micrographs were compared. Representative micrographs were shown in Figs 55-57 inclusive. In each group, cell shape ranges from compact and spherical to markedly flattened, with intermediate shapes including elongated, triangular, stellate or irregularly shaped. A higher proportion of control macrophages are still spherical after 48 hours in culture than in either of the other two groups, whereas N.asteroides and N.brasiliensis-activated macrophages have become elongated or very irregular in shape. Well-spread cells in both the Nocardia-activated macrophage cultures appear to be more markedly flattened than those in control cultures - in the latter the dome-shaped central nuclear region is very prominent.

5.8.1 SEM OF CONTROL MACROPHAGES

Shape and surface morphology of control macrophages was, in general, similar to that described in the literature and summarized in section 5.6.2. Representative examples of control macrophage shape and surface characteristics are shown in the micrographs Figs 58-63 inclusive.

Inspection of Figs 58, 59 reveals that control macrophages have spread to a certain extent, have assumed spherical or irregular shapes, have a ruffled surface and numerous peripheral radiating filopodia. Breaking off of filopodia is an artefact due to cell shrinkage on dehydration. Membrane structure varies from ruffled and ridged, eg Fig 62, to highly microvillous (Fig 61), to smooth with raised ruffled nuclear area (Fig 63). This micrograph shows an elongated macrophage with long pseudopodia

flanked by two well-spread cells. Extensive filopodia are a commonly noted characteristic of control macrophages. The micrograph (Fig 60) shows a spherical cell which has started to spread on the glass substrate by extending a veil of cytoplasm beneath the dome-shaped nuclear area. The cell has extensive filopodia and microvilli.

Macrophage size was estimated by measuring the diameter of spherical cells or the length of the long axes of triangular or elongated cells. Median values and their 95% confidence limits were determined and these data and are shown in Table 119.

5.8.2 SEM OF N.ASTEROIDES-ACTIVATED MACROPHAGES

Although, as stated previously, there was a clear distinction between morphology of N.asteroides-activated and control macrophages using low and high power SEM, no clear distinction was apparent between cells harvested 2, 7, 13 or 21 days post-inoculation, ie time post-inoculation did not appear to influence surface morphology. N.asteroides-activated macrophages exhibited characteristic surface ruffles and ridges, eg the spherical cell in Fig 64; and this was the most commonly noted surface structure for spherical cells, although some spreading spheres were covered with microvilli and occasionally blebs (Fig 65). This cell also has extensive radiating peripheral filopodia. Comparison of spherical cells in control and N.asteroides -activated macrophages revealed that consistent characteristics of activated cells were more prominent and larger undulating membrane ruffles, deeper folds, prominent flaplike-protrusions

(Figs 75, 80, 81) and in general a greater variety of membrane processes over the surface were noted.

N.asteroides-activated macrophages assumed a wide range of shapes. Extensive spreading was a feature of these cultures with cells becoming highly polarized or elongated with long pseudopodia terminating in broad ruffled cytoplasmic veils (Fig 66) or in numerous radiating slender filopodia (Fig 67). Cells assumed stellate or triangular forms with pseudopodia or broad veil-like lamellipodia and long filopodia (Figs 67, 68). As in control cultures, spread macrophages had fewer surface ruffles than the spherical cells, however, N.asteroides-activated well-spread cells appeared to be smoother than controls which were similar, but less extreme conformations. Flattened well-spread macrophages (part shown in Fig 67) had smooth peripheral veils of cytoplasm, subplasmalemmal sphericles and ruffled perinuclear area. N.asteroides-activated macrophages were generally associated with filopodia, eg the spreading cell in Fig 71.

Macrophage size was estimated in exactly the same way as for control cells and median values and their 95% confidence limits calculated, and shown in Table 119. N.asteroides-activated cells were thus similar in size to controls when spherical but spread cells appeared to be larger than controls, from the median values (Table 119). This difference was, however, not statistically significant (Mann Whitney U Test).

5.8.3 SEM OF N.BRASILIENSIS-ACTIVATED MACROPHAGES

Examination of SEM micrographs of macrophages harvested from mice 2, 7, 13 and 21 days post-inoculation with N.brasiliensis did not reveal any distinctive differences in surface morphology which could be related to duration of infection. The most marked effect of prior inoculation with N.brasiliensis on macrophage morphology was the extremes of shape of cells. The contrast in shape is easily seen by comparing the low power micrographs of control and N.brasiliensis-activated cells (Fig 58, 72).

Cell shape varied from spherical (Fig 73) to markedly flattened (Figs 77, 78, 75) to stellate or highly irregular in shape (Figs 88, 89, 86). Characteristic complex surface ruffles and ridges and in some cases microvilli were present on cells, with surface ruffling being far more prominent in compact spherical cells than in well-spread cells (Johnson and Davies, 1981).

Surface projection of spherical N.brasiliensis-activated macrophages were larger and more prominent and present in a greater variety than those of control cells. Characteristic protrusions were undulating extensive thick ruffles and ridges with deeper folds between them than controls; some cells had large flanges of membrane perpendicular to the cell surface and others showed larger indented bulbous projections (Fig 70, compared to Fig 73).

In spread cells surface ruffling was associated with the raised

central nuclear area. Subplasmalemmal sphericles were evident in markedly flattened macrophages (Fig 76) as well as craters in the smooth peripheral cytoplasm of some flattened cells. N.brasiliensis-activated macrophages showed greatly enhanced membrane activity evidenced by extensive flattening of cells with ruffled veil edges (Figs 74, 57) and frequent filopodia. Extensive lamellipodia and pseudopodia were characteristic, these features terminating in ruffled veil edges or numerous radiating filopodia (Figs 78, 75, 77). Highly polarized long thin cells were commonly seen, with the characteristic raised ruffled central area and pseudopodia terminating in veils of cytoplasm or radiating filopodia. Comparison of micrographs of irregularly spread control and N.brasiliensis-activated macrophages revealed that in general fewer surface projections were associated with activated cells than control cells.

Macrophage size was estimated and median values and their 95% confidence limits calculated, and shown in Table 119. The median diameter of spherical cells was similar to that of controls and N.asteroides-activated macrophages. N.brasiliensis-activated cells when spread were similar in size to N.asteroides-activated macrophages but appeared to be larger than control cells, from the median values. This difference was however, not statistically significant (Mann Whitney U Test), except for control cells compared to N.brasiliensis-activated cells in the elongated from (significant at the 0.03 level).

5.8.4 GENERAL COMMENTS ON SEM CHARACTERISTICS

The most marked morphological difference between control and Nocardia-activated macrophage cultures when examined using SEM was the variety and extremes of shape assumed by the Nocardia-activated macrophages. Although both control and Nocardia-activated macrophages spread extensively, Nocardia-activated macrophages appeared to be more flattened than controls.

Nocardia-activated macrophages appeared to spread more extensively than did control cells, gauged from cell size, however this difference was not significant, except for control and N.brasiliensis-activated cells in the elongated form. These results concur with those of the phase-contrast microscopy study.

A further characteristic, which was more difficult to assess, was that fewer filopodia appeared to be associated with Nocardia-activated macrophages than with control cells. It is obvious from the micrographs presented that Nocardia-activated macrophages were often associated with extensive filopodia formation but assessment of over 200 micrographs revealed that irregularly shaped cells (eg Fig 78), markedly elongated and triangular or stellate cells had fewer filopodia than controls in similar but less extreme conformations. The long pseudopodia of highly polarized Nocardia-activated macrophages appeared to terminate more frequently in broad undulating ruffled cytoplasmic veils (eg Fig 66) than in filopodia (eg the control macrophages shown in Fig 79).

There did not appear to be any difference in the morphology of N.asteroides and N.brasiliensis-activated macrophages. In both groups extremes of shape were evident and no characteristic surface features could be associated with either group. In addition, duration of infection did not appear to influence cell surface morphology.

5.9 DISCUSSION

Control, unstimulated macrophages show morphologic evidence of a high degree of activity with numerous ruffles, ridges and microvilli traversing the cell surface in a random fashion; and the presence of long cytoplasmic extensions. This finding in in agreement with that of Polliack and Gordon (1975). Numerous fine radiating filopodia associated with control cells were present. The presence of numerous filopodia has been associated with stimulated or activated macrophages (Polliack and Gordon, 1975; Reed, Douglas and Speer, 1982). Macrophage stimulation occurs as a result of the presence of serum in the culture medium and cell surface structures are consequently modified (Albrecht, Jordan and Hong, 1978; Cohn and Benson, 1965b). Morland and Kaplan (1977) also found that formation of filopodia occured when cells were cultured in serum- containing medium.

Macrophage surface structure has been shown to be dependent on the degree of macrophage stimulation (Daems, 198Ø : 83; Albrecht, Jordan and Hong, 1978). Stimulated cells are described as being larger and flatter than unstimulated cells with greater

variation in cell shape and surface projections, as well as being associated with pseudopodia, broad lamellipodia with rolled edges and filopodia (Polliack and Gordon, 1975; Carr, 1980 : 266).

Nocardia-activated macrophages differed from controls in several respects when their morphology was compared using SEM. Activated macrophages appeared to be larger than control cells when spread, they showed greatly increased membrane activity manifested by more extensive spreading, the presence of larger and more varied ruffles and membranous micro-projections (spherical cells) and the more frequent appearance of lamellipodia with prominent rolled edges and pseudopodia. In the literature, stimulated macrophages are associated with more extensive and more prominent ruffles and other membranous processes giving the stimulated cell a rougher appearance (Carr et al, 1969; Albrecht et al, 1972; Polliack and Gordon, 1975). Lamellipodia formation was more extensive and occurred more frequently in stimulated or activated phagocytes (Polliack and Gordon, 1975; Biemesderfer, 1978).

Nocardia-activated macrophages were very much more pleomorphic in nature than control cells, adopting extremes of shapes. Greater variation in shape is a characteristic of stimulated macrophages (Polliack and Gordon, 1975; Carr, Clarke and Salsbury, 1969; Carr, 1980 : 266).

A variety of shapes were adopted by macrophages in both control

and Nocardia-activated macrophage cultures but the incidence of the different shapes differed markedly in the different cultures, with Nocardia-activated macrophages characterized by markedly flattened, stellate or elongated macrophages whereas control cultures showed large numbers of spherical cells and spread cells were not as flattened as Nocardia-activated macrophages. Stimulated cells are described as being larger and flatter than unstimulated cells (Papadimitriou, Finlay-Jones and Walters, 1973) and it has been suggested that flattening of rounded cells is a result of cell activation (Adler et al, 1979). Sphericles (lysozomes) were noted in the smooth cytoplasmic extensions of flattened Nocardia- activated macrophages and increased numbers of lysozomes are associated with activated cells (section 5.1.1).

Irregularly-shaped, well-spread Nocardia-activated macrophages had numerous peripheral processes but lacked extensively ruffled membranes. This same appearance was described for Brucella-activated macrophages (Albrecht et al, 1978a). This phenomenon may perhaps be explained by the concept that well-spread macrophages use the extensive membrane folds of spherical cells to supply extra membrane required in spreading (Newell, 1980 : 256). Since Nocardia- activated cells spread more extensively than control cells it follows that they would have fewer surface ruffles than control macrophages.

Beaman (1977) found that macrophage response to Nocardia depended on the strain of Nocardia being studied. In this study

morphological characteristics could not be strictly associated with either N.asteroides or N.brasiliensis. Beaman's experimental system differs from that used here in that macrophages were exposed to virulent and less virulent Nocardia asteroides in vitro and this could explain the lack of correlation of the data.

In conclusion, morphology of control and Nocardia-activated macrophages when compared using SEM is different. Nocardia-activated macrophages display many of the characteristics of activated macrophages as detailed in the literature and on the basis of the morphology studies using phase contrast and SEM, may be considered to be activated.

5.10 FIGURES

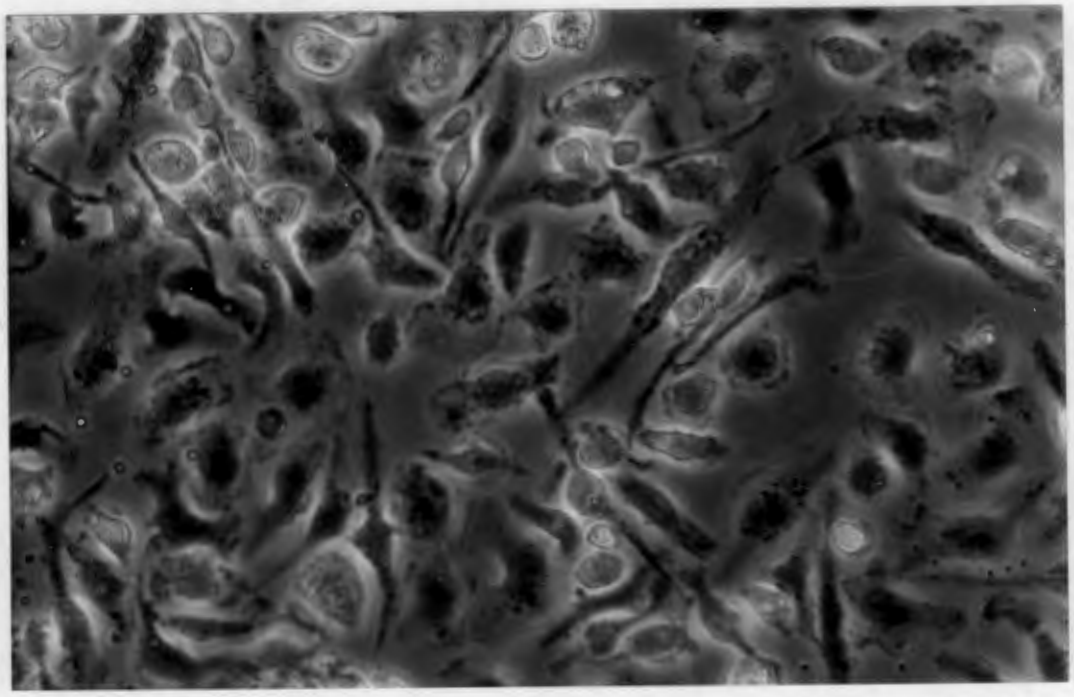

FIG. 43. Phase-contrast photomicrograph of living macrophages from mice inoculated 2 days previously with N.asteroides. Cells were incubated for 13.5 hours (magnification x 320).
Cultures shown in figs 43-45 inclusive were incubated under the same conditions and run concurrently.

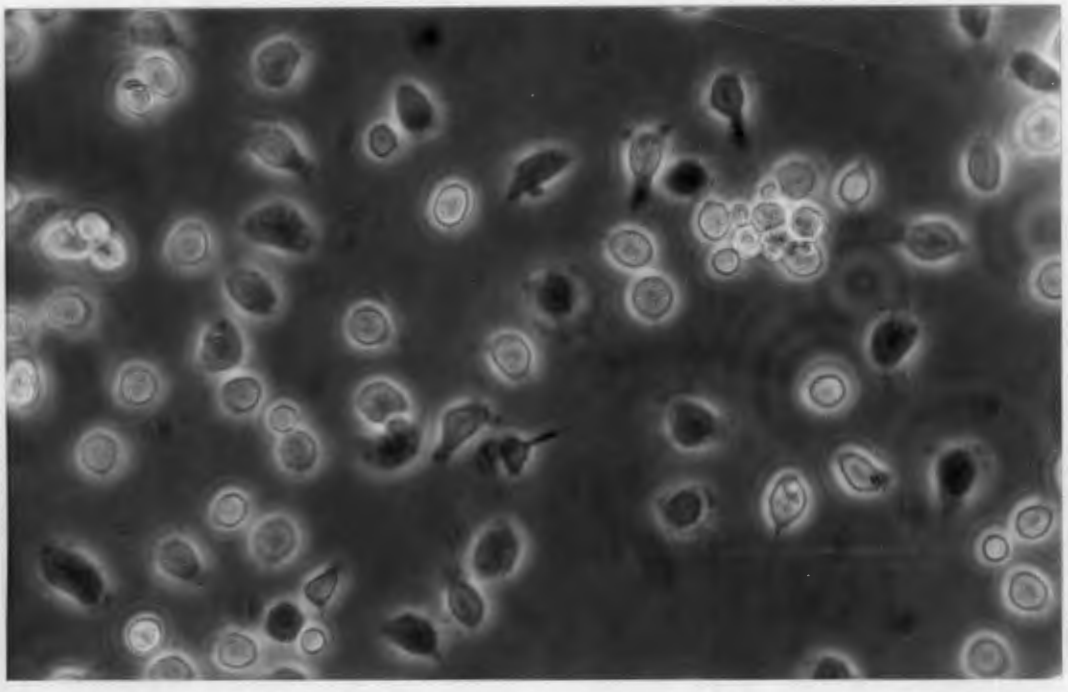

FIG. 44. Phase-contrast photomicrograph of living macrophages from mice inoculated 2 days previously with saline only. Cells were incubated for 13.5 hours (magnification x 320).

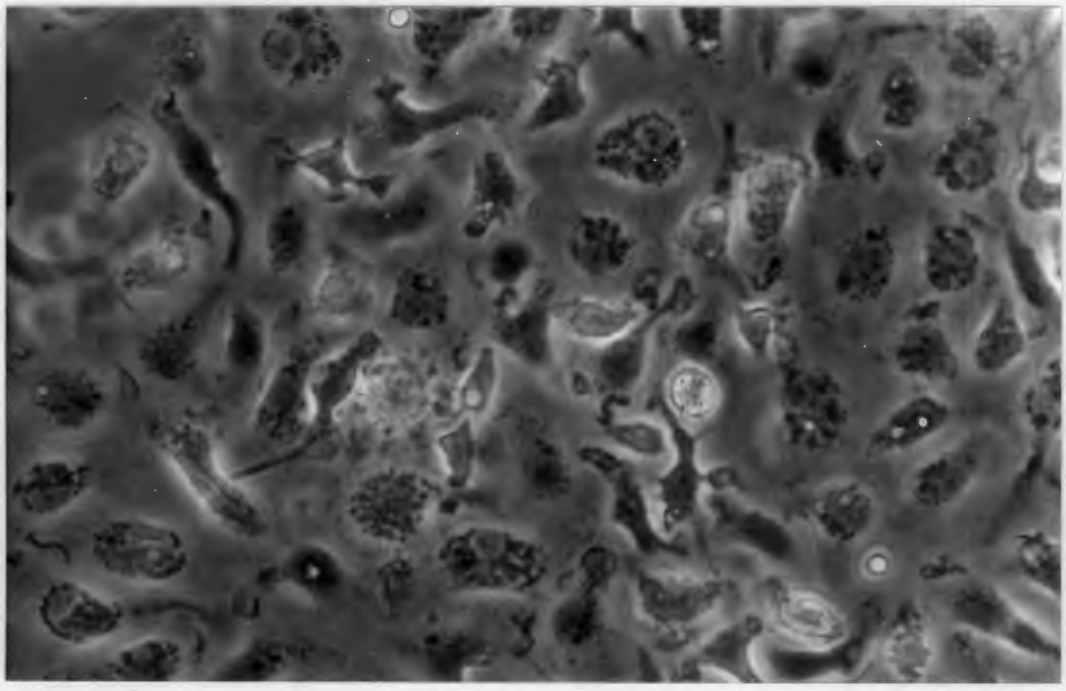

FIG. 45. Phase-contrast photomicrograph of living macrophages from mice inoculated 2 days previously with N.brasiliensis. Cells were incubated for 13.5 hours (magnification x 320).

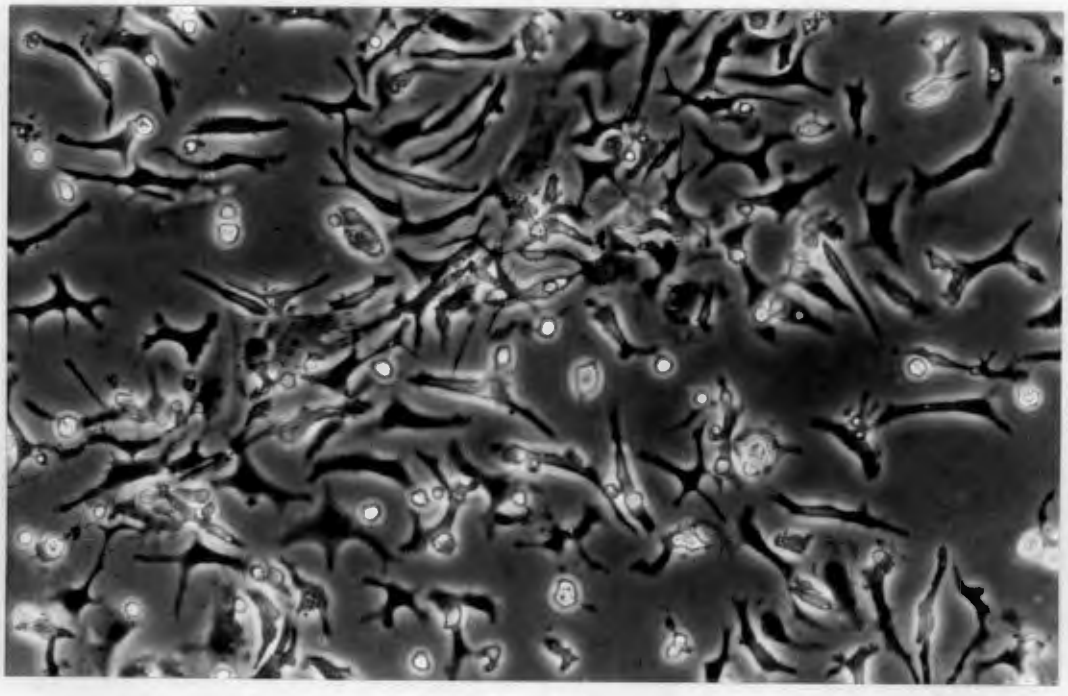

FIG. 46. Phase-contrast photomicrograph of living macrophages from mice inoculated 7 days previously with N.asteroides. Cells were incubated for 46 hours (magnification x 200).

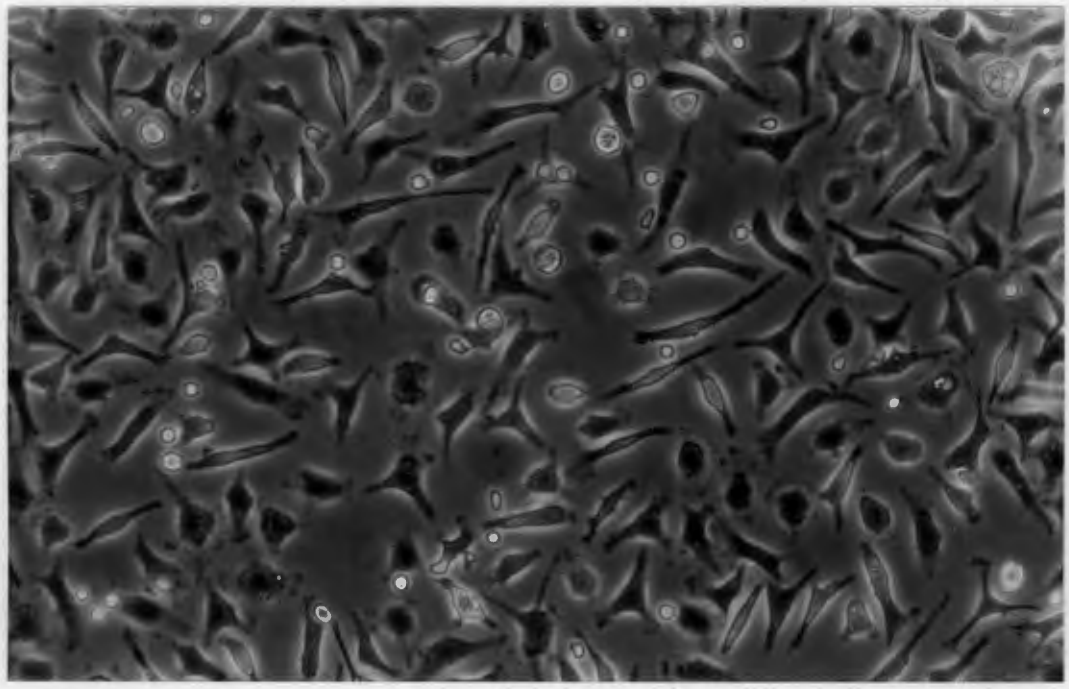

FIG. 47. Phase-contrast photomicrograph of living macrophages from mice inoculated 7 days previously with saline only. Cells were incubated for 72 hours (magnification x 200).
Cultures shown in figs 47 and 48 were incubated under the same conditions and run concurrently.

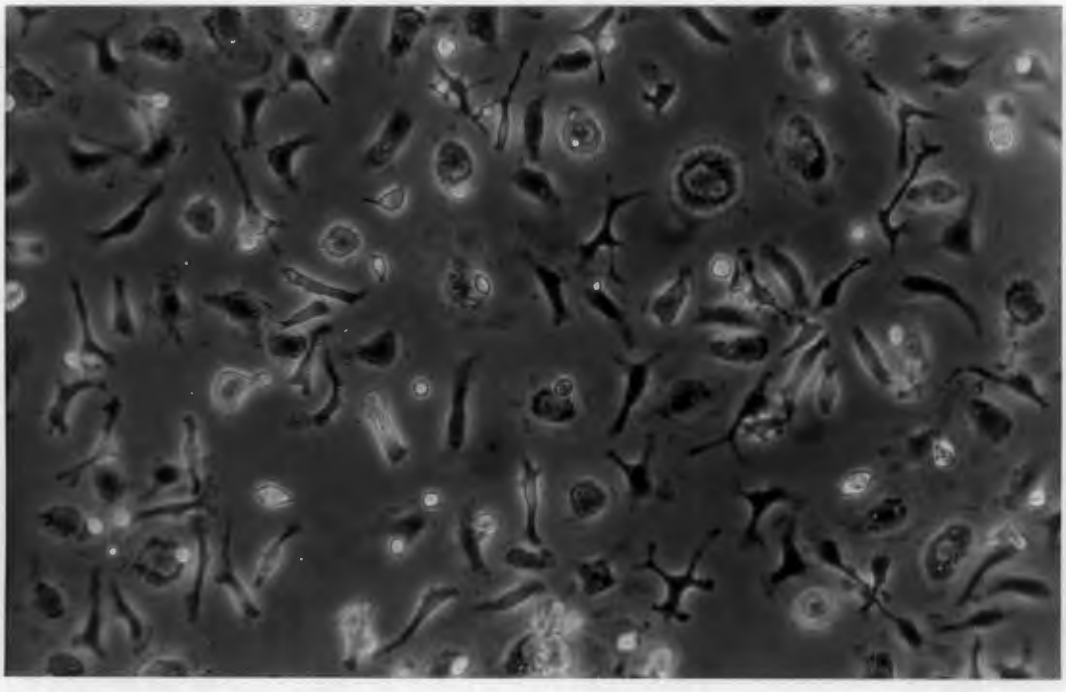

FIG. 48. Phase-contrast photomicrograph of living macrophages from mice inoculated 7 days previously with N.brasiliensis. Cells were incubated for 72 hours (magnification x 200).

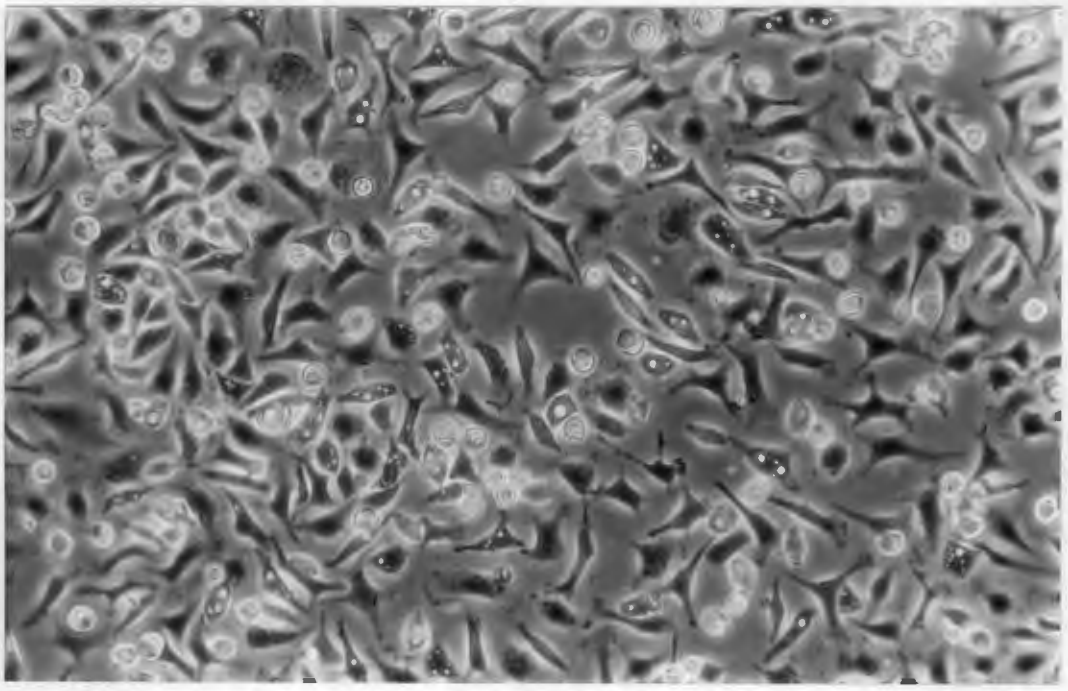

FIG. 49. Phase-contrast photomicrograph of living macrophages from mice inoculated 13 days previously with saline only. Cells were incubated for 42 hours (magnification x 200).
Cultures shown in figs 49 and 50 were incubated under the same conditions and run concurrently.

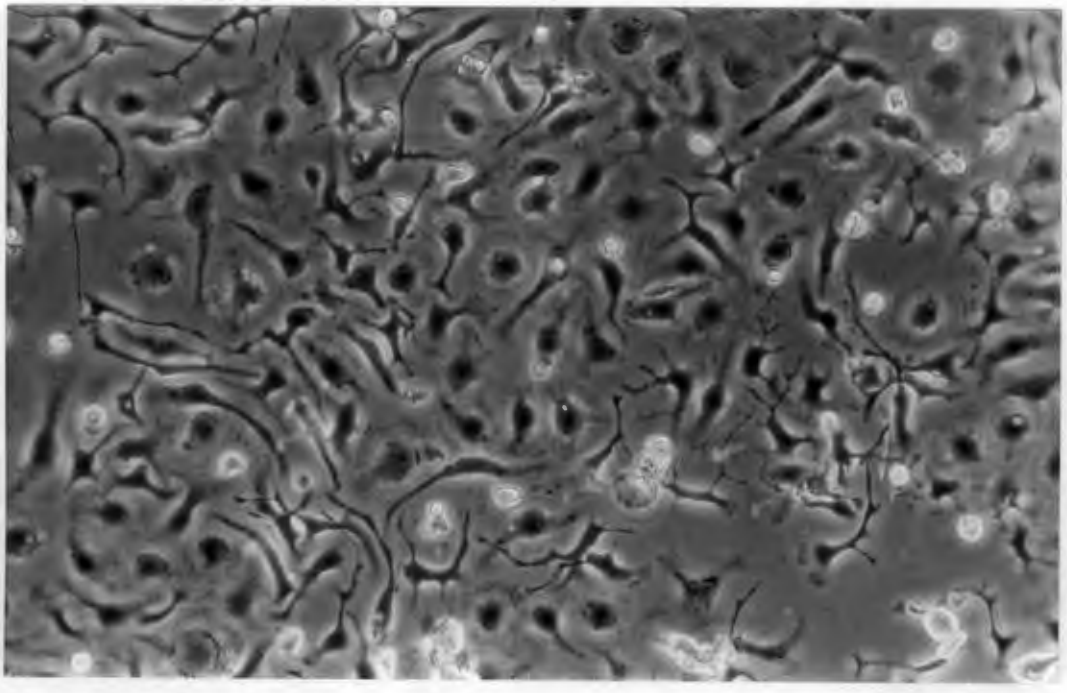

FIG. 50. Phase-contrast photomicrograph of living macrophages from mice inoculated 13 days previously with N.asteroides. Cells were incubated for 42 hours (magnification x 200).

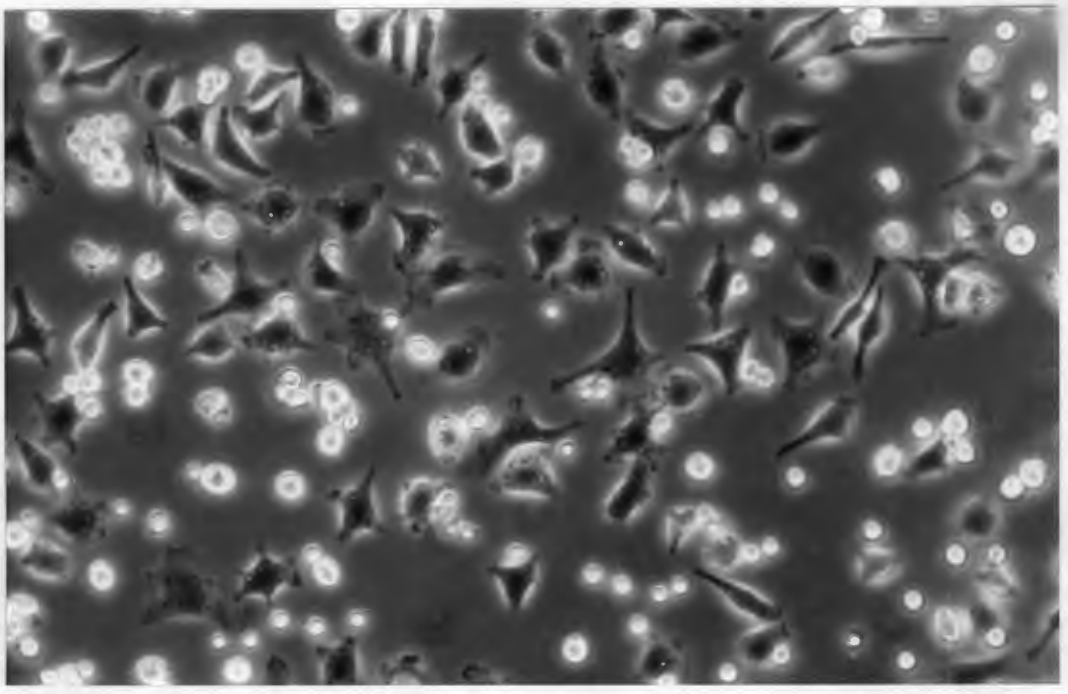

FIG. 51. Phase-contrast photomicrograph of living macrophages from mice inoculated 13 days previously with saline only. Cells were incubated for 2.5 hours (magnification x 200).
Cultures shown in figs 51 and 52 were incubated under the same conditions and run concurrently.

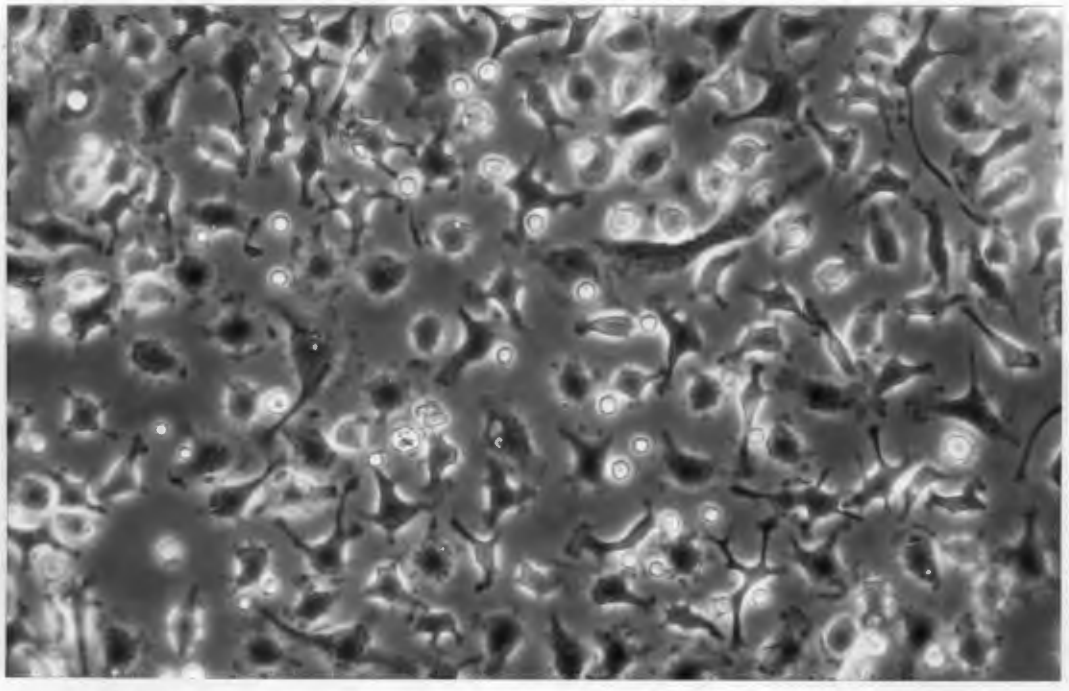

FIG. 52. Phase-contrast photomicrograph of living macrophages from mice inoculated 13 days previously with N.brasiliensis. Cells were incubated for 2.5 hours (magnification x 200).

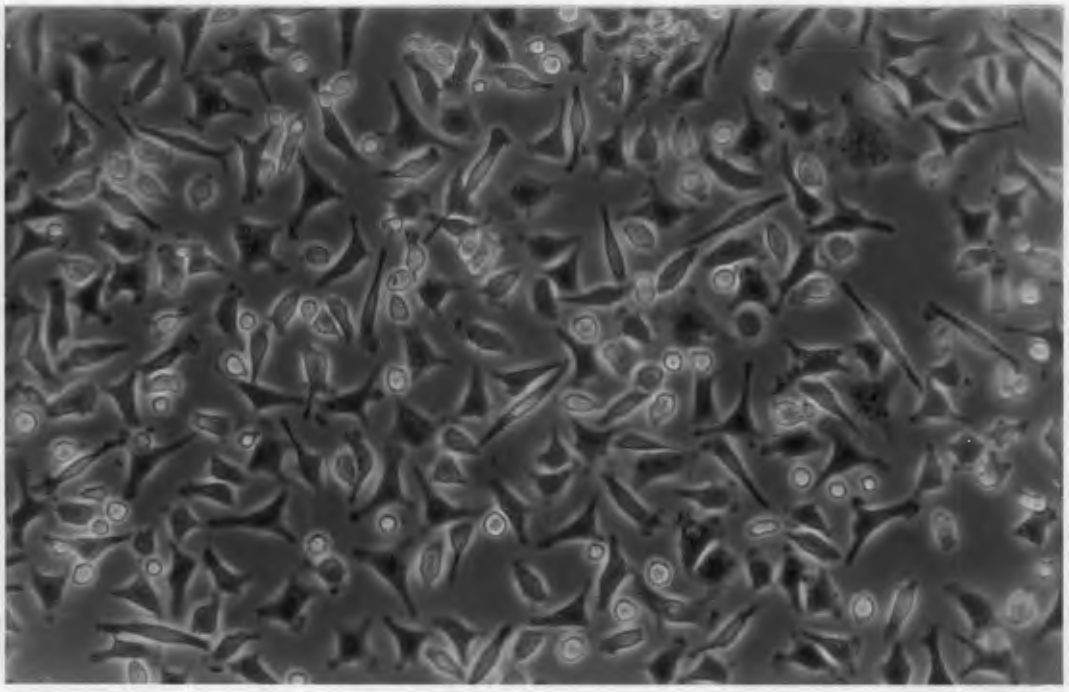

FIG. 53. Phase-contrast photomicrograph of living macrophages from mice inoculated 21 days previously with saline only. Cells were incubated for 24 hours (magnification x 200).
Cultures shown in figs 53 and 54 were incubated under the same conditions and run concurrently.

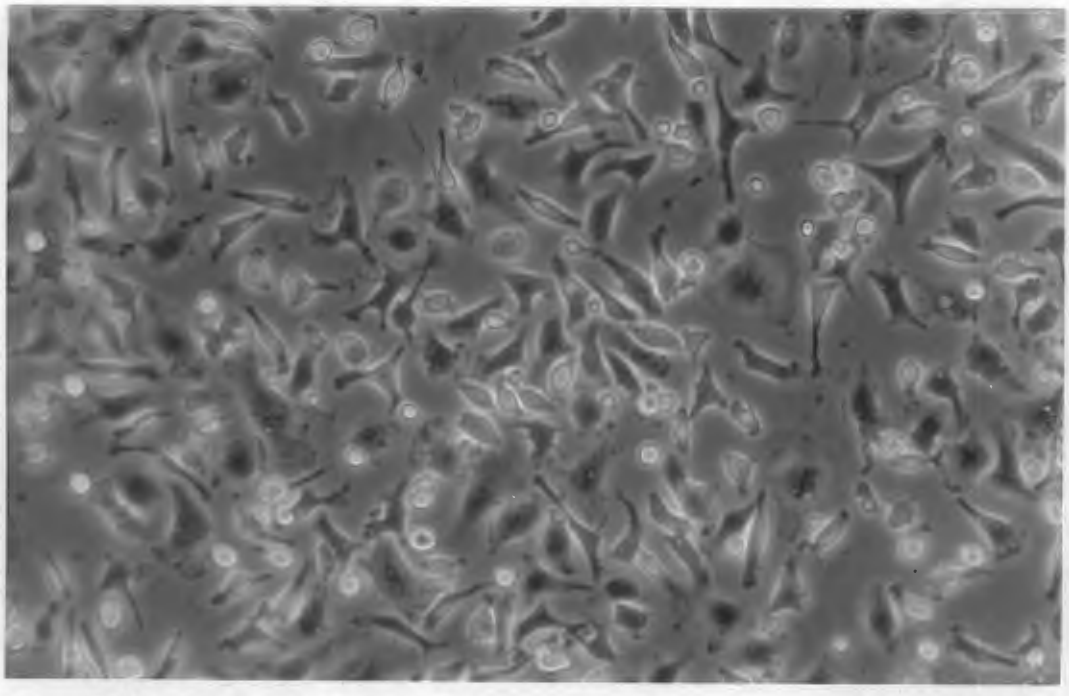

FIG. 54. Phase-contrast photomicrograph of living macrophages from mice inoculated 21 days previously with N.asteroides. Cells were incubated for 24 hours (magnification x 200).

ALL CULTURES WERE INCUBATED FOR 40 HOURS

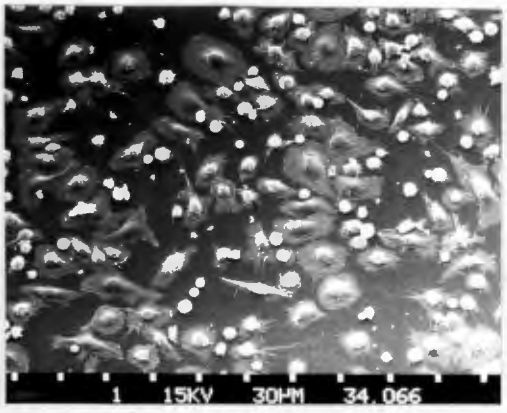

FIG. 55
Scanning electron micrograph of macrophages from mice inoculated 21 days previously with saline only (magnification x 310).

FIG. 56
Scanning electron micrograph of macrophages from mice inoculated 13 days previously with N.asteroides (magnification x 350).

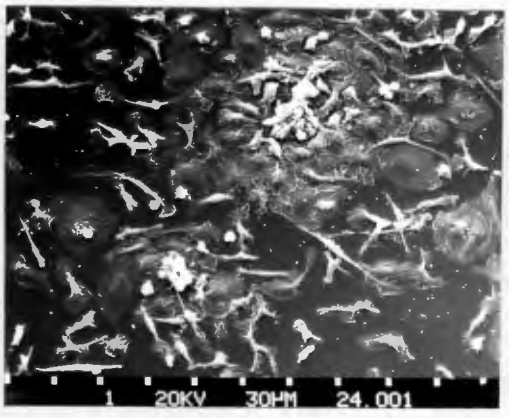

FIG. 57
Scanning electron micrograph of macrophages from mice inoculated 7 days previously with N.brasiliensis (magnification x 305).

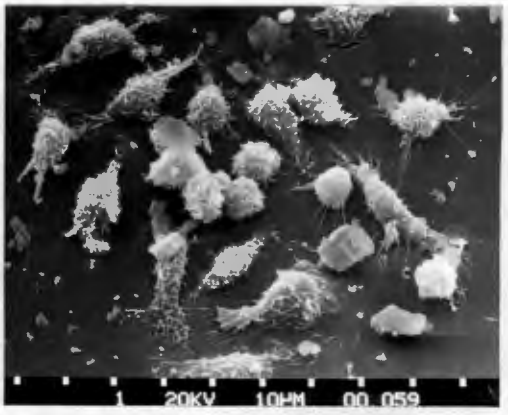

FIG. 58
Scanning electron micrograph of macrophages from mice inoculated 21 days previously with saline only (magnification x 1150).

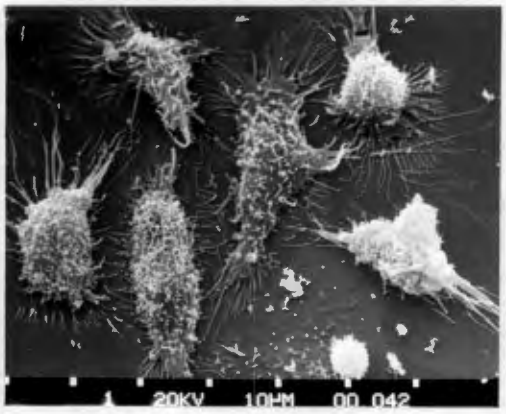

FIG. 59
Scanning electron micrograph of macrophages from mice inoculated 21 days previously with saline only (magnification x 1540).

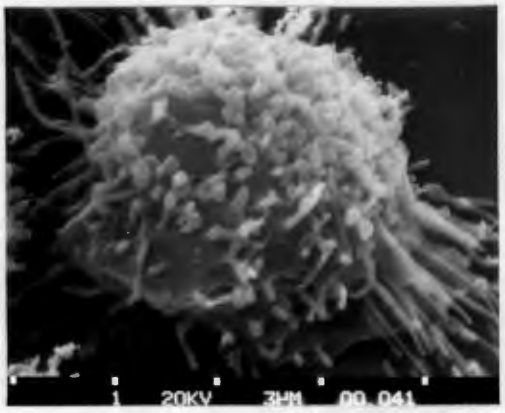

FIG. 60
Scanning electron micrograph of a spherical cell with extensive filopodia and microvilli. Mice were inoculated 21 days previously with saline only (magnification x 7700).

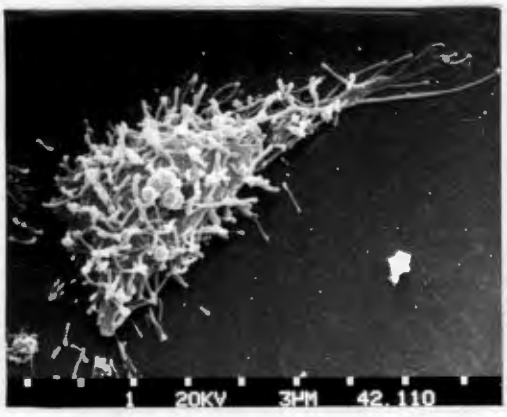

FIG. 61
Scanning electron micrograph of a highly microvillous triangular-shaped macrophage harvested from mice inoculated 13 days previously with saline only (magnification x 3500).

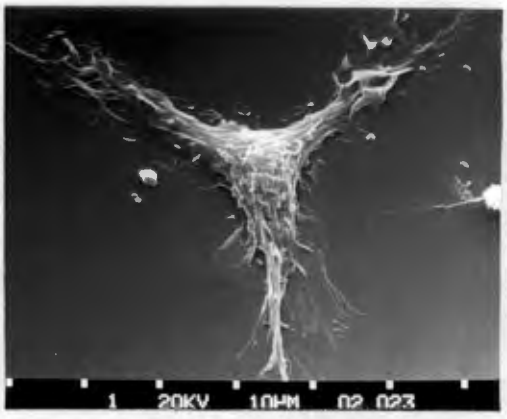

FIG. 62
Scanning electron micrograph of a triangular-shaped macrophage with ruffled and ridged membranes. Mice were inoculated 7 days previously with saline only (magnification x 1710).

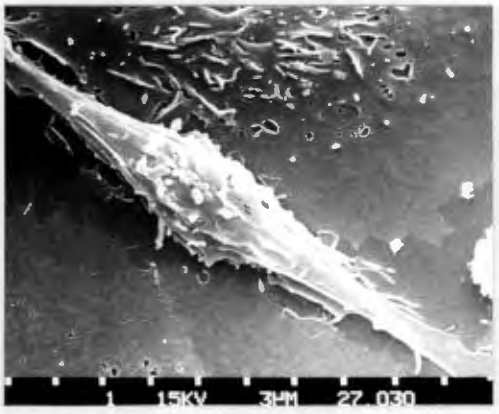

FIG. 63
Scanning electron micrograph of an elongated macrophage with long pseudopodia and raised ruffled nuclear area; between two flattened, well-spread cells. Mice were inoculated 13 days previously with saline only (magnification x 3070).

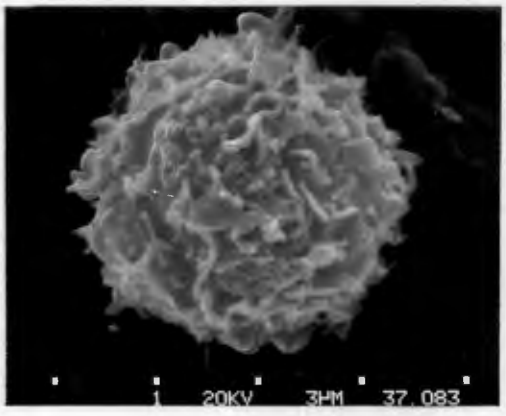

FIG. 64
Scanning electron micrograph of a spherical macrophage with surface ruffles and ridges. Mice were inoculated 7 days previously with *N. asteroides* (magnification x 6600).

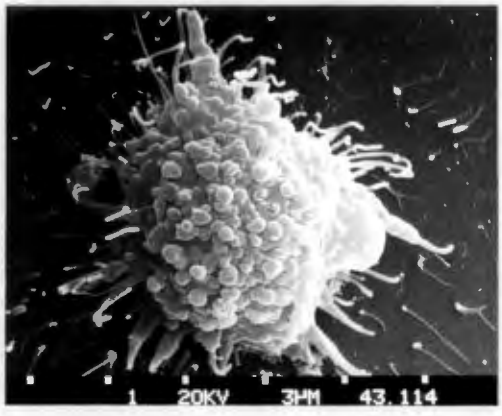

FIG. 65
Scanning electron micrograph of a spreading spherical macrophage showing microvilli and blebs as well as radiating peripheral filopodia. Mice were inoculated 13 days previously with *N. asteroides* (magnification x 5100).

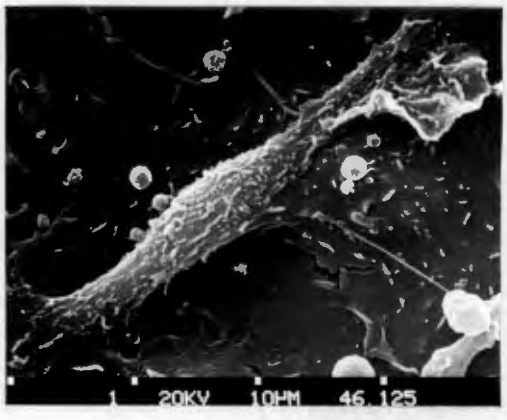

FIG. 66
Scanning electron micrograph of an elongated macrophage with long pseudopodia terminating in broad ruffled cytoplasmic veils. Mice were inoculated 2 days previously with *N. asteroides* (magnification x 2410).

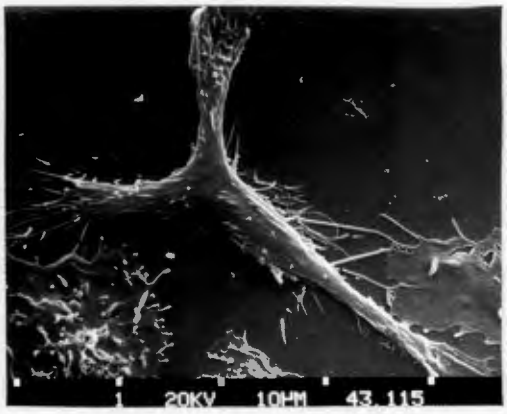

FIG. 67
Scanning electron micrograph of a triangular-shaped macrophage with pseudopodia terminating in numerous radiating slender filopodia (13 day N.asteroides infection; magnification x 2000).

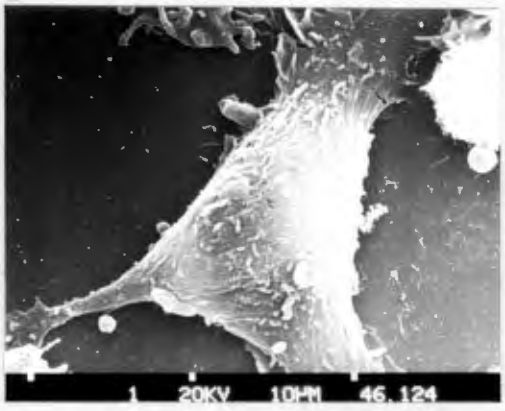

FIG. 68
Scanning electron micrograph of a triangular-shaped macrophage with fairly smooth membranes and broad lamellipodia (2 day N.asteroides infection; magnification x 3140).

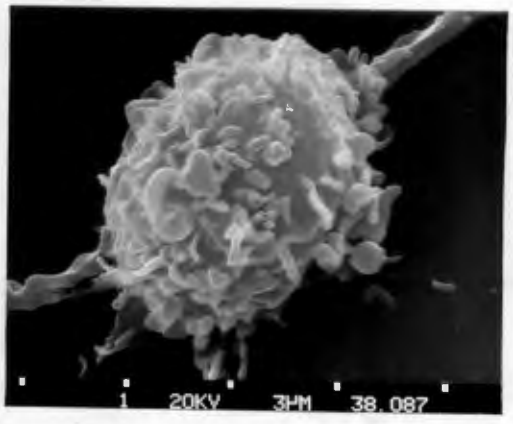

FIG. 69
Scanning electron micrograph of a spherical macrophage showing prominent membrane ruffles and flap-like protrusions (7 day N.asteroides infection; magnification x 6800).

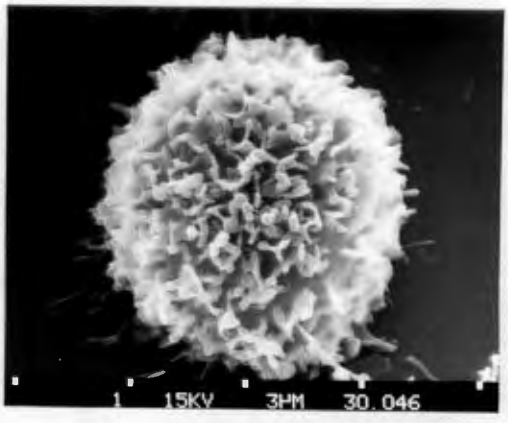

FIG. 70
Scanning electron micrograph of a spherical macrophage showing ruffled membranes. Mice were inoculated 2 days previously with saline only (magnification x 7500).

FIG. 71

Scanning electron micrograph of a spreading cell showing filopodia and membrane ruffles (7 day N.asteroides infection; magnification x 4100).

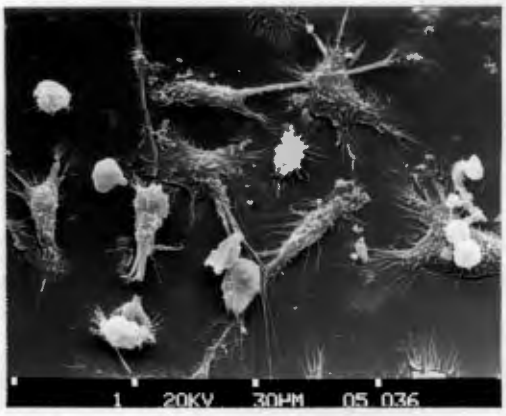

FIG. 72
Scanning electron micrograph of macrophages harvested from mice incubated 7 days previously with N.brasiliensis. Micrograph shows variation in shape of cells (magnification x 910).

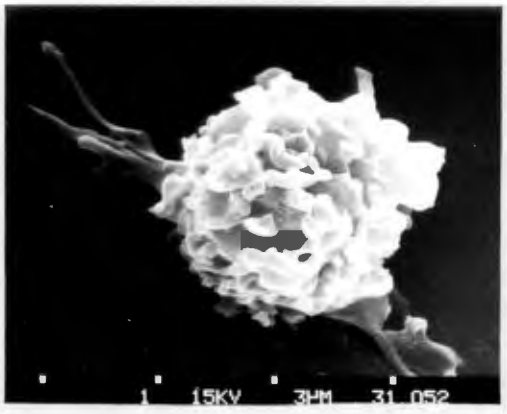

FIG. 73
Scanning electron micrograph of a spherical macrophage showing prominent surface ruffles (2 day N.brasiliensis infection; magnification x 7500).

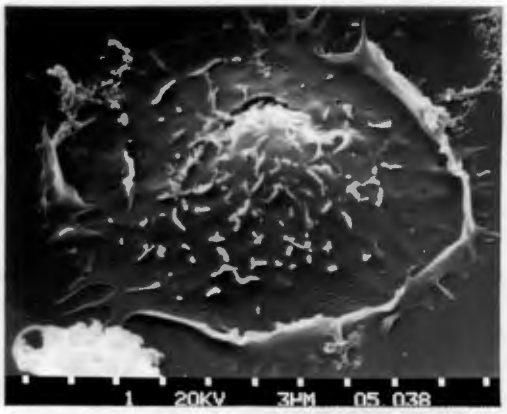

FIG. 74
Scanning electron micrograph of a flattened macrophage showing raised ruffled nuclear pole and smooth cytoplasmic veils (enhanced membrane activity shown by increased spreading and frequent filopodia). Mice were inoculated 7 days previously with N.brasiliensis (magnification x 3500).

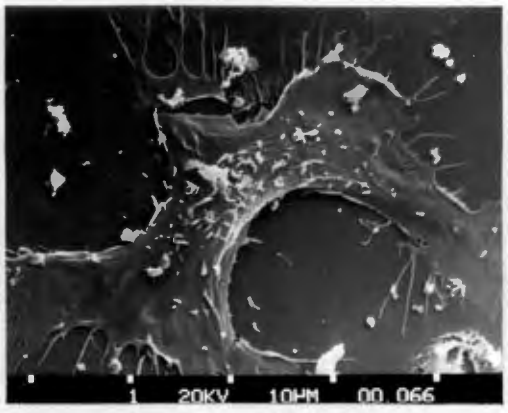

FIG. 75
Scanning electron micrograph of a well-spread, irregularly shaped macrophage which is markedly flattened with smooth cytoplasmic veils, some ruffling over the nuclear pole, broad veils and numerous filopodia (21 day N.brasiliensis infection; magnification x 1960).

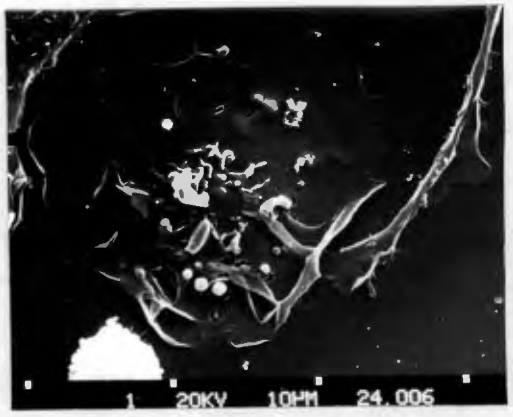

FIG. 76
Scanning electron micrograph of a markedly flattened macrophage showing smooth cytoplasmic veils with subplasmalemmal sphericals and ruffled area over the nuclear pole (7 day N.brasiliensis infection; magnification x 2820).

FIG. 77
Scanning electron micrograph of a triangular-shaped macrophage showing extensive pseudopodia and filopodia (2 day N.brasiliensis infection; magnification x 4100).

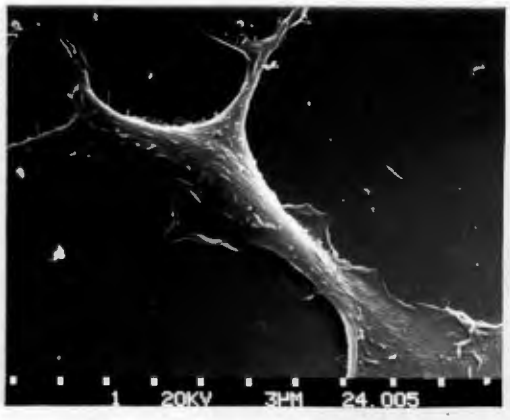

FIG. 78
Scanning electron micrograph of an elongated, irregularly shaped macrophage with extensive lamellipodia and pseudopodia (7 day N.brasiliensis infection; magnification x 7500).

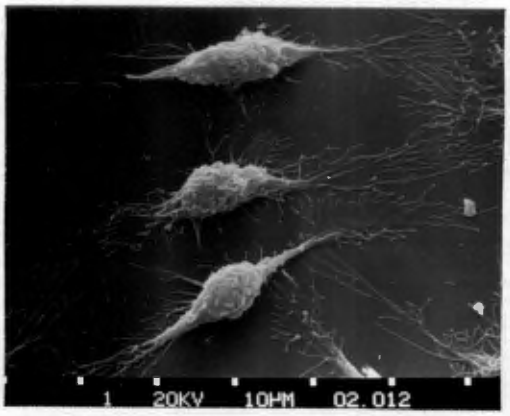

FIG. 79
Scanning electron micrograph of 3 control macrophages showing ruffled membranes and extensive filopodia. Mice were inoculated 2 days previously with saline only (magnification x 1740).

5.11 TABLES

TABLE 117

Protein content as an expression of cell size. Macrophages harvested from mice 2 days post-inoculation. Cells washed after 2 hours in culture to remove non-adherent cells then inoculated for a further 10 hours before assay (1 x 10^6 macrophages plated initially).

	µg cell protein	
	in 50 µl sample	in 1 x 10^6 macrophages
Control	2.46	49.2
	3.22	64.4
	4.57	91.4
	5.26	105.2
N.asteroides-Activated	5.76	115.2
	4.21	84.2
	2.93	56.6
	3.52	70.4
N.brasiliensis-Activated	3.24	64.8
	3.07	61.4
	7.06	141.2
	5.84	116.8

TABLE 118

Nomenclature of macrophage surface projections observed by SEM

Lamellipodium	Extended flap-like process, veil or large undulating membrane, generally smooth but may be interrupted by intracytoplasmic spherules or ruffles.
Pseudopodium	Broad, footlike process extended outward along a substrate from the central nuclear cell area.
Filopodium	Long, thin, thread-like process projected from the cell along the substrate, often appearing as an attachment extension and generally beginning at a cell substrate junction.
Ruffle	Membrane extension appearing as a wavy, undulating fold roughly perpendicular and found anywhere on a cell surface.
Ridge	Broad-based narrow surface fold.
Microvilli	Short, cylindrical or finger-like membrane extensions roughly perpendicular to the cell surface of uniform diameter and variable length.
Bleb	Hemispherical or bulbous projections extended outward from the cell surface.
Cobblestone	A membrane patch which is round and platelike and which is layered against the cell surface, with the appearance of a collapsed ruffle. Cells with cobblestones have a layered appearance.
Crater	A rounded indentation or small depression in smooth peripheral area.
Lacuna	Small opening.
Spherule	Intracellular or intracytoplasmic spherical body, may represent a subplasmalemmal cytoplasmic organelle, probably a lysozome or lipid body.

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Warfel, Elberg 1970

TABLE 119

Macrophage size from SEM micrographs, estimated by measuring the diameter of spherical cells or the length of the long axes of triangular or elongated cells : median values and their 95% confidence limits (data expressed in µm).

		Macrophage Shape			
		Sphere	Flattened	Triangle	Elongated
		n=16	n=2	n=9	n=8
Control	M	9.245	30.15	18.79	44.27
	LL	8.245	8.2	16.06	30.54
	UL	10.3	52.10	30.5	58.6
		n=9	n=0	n=6	n=10
Na	M	8.49		25.1	58.9
	LL	7.53		21.99	51.1
	UL	9.99		28.21	66.8
		n=11	n=6	n=9	n=21
Nb	M	7.74	29.4	27.01	56.55
	LL	6.77	26.83	20.61	50.8
	UL	8.805	31.97	35.6	61.5

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SECTION IV

CONCLUSIONS

CHAPTER 6

DEVELOPMENTS IN UNDERSTANDING THE PATHOGENESIS OF *NOCARDIA ASTEROIDES* AND *NOCARDIA BRASILIENSIS* INFECTIONS

6.1 DISCUSSION

6.2 SUMMARY OF FINDINGS OF THIS STUDY

6.3 REFERENCES

Chapter 6

DEVELOPMENTS IN UNDERSTANDING THE PATHOGENESIS OF *NOCARDIA ASTEROIDES* AND *NOCARDIA BRASILIENSIS* INFECTIONS

6.1 DISCUSSION

There are several reasons why our efforts in this study have been focused on the macrophage:

1. The histological features of experimental *Nocardia* infection suggested an important role of the macrophage in normal host defence against the organisms;

2. The macrophage plays a pivotal role in defence against related infections such as tuberculosis, leprosy, leishmaniasis and listeriosis;

3. In the literature on experimental *Nocardia* infections and on *in vitro* studies it has been shown that activated macrophages are important in host defence against *Nocardia*.

When this study was begun, the role of the macrophage in host resistance to *Nocardia* was unclear. The investigation was prompted by an interest in determing whether differences noted in experimental murine *Nocardia* infections might be explained in part or in whole by differences in macrophage function. Also, few investigations have directly examined the effects of *Nocardia* infection on macrophage function. Black et al (1983, 1985)

studied the effect of N.asteroides infection on macrophage intracellular levels of the acid hydrolase acid-phosphatase; apart from this work, biochemical aspects of macrophage function in Nocardia infections do not appear to have been reported in any detail. Other aspects of macrophage function in Nocardia infection that have been studied are phagocytosis (Beaman, 1979; Davis-Scibienski and Beaman, 1980 a and c; Black et al, 1983, 1985), macrophage migration inhibition (Sundararaj and Agarwal, 1977a) and macrophage aggregation (Sundararaj and Agarwal, 1977a).

In looking at macrophage function in experimental Nocardia infections, we clearly had to examine biochemical, functional, as well as morphological aspects and for this reason the various parameters reported were selected.

It is known that facultative intracellular parasites are able to survive and multiply within macrophages. Their capacity to resist the microbicidal mechanisms of these cells is a major factor in their pathogenicity (Mauel, 1982). The parasites appear to survive by one or both of two mechanisms: (i) they have acquired the ability to avoid or counteract the effects of lysozomal enzymes and (ii) they may gain access to host cells without stimulating the respiratory burst. There are at least three mechanisms whereby intracellular parasites are able to avoid the destructive effects of lysozomal constituents: inhibition of phagosome-lysozome fusion, resistance to lysozomal constituents and escape from phagolysozomes. Resistance to

lysozomal constituents may possibly be achieved by presence of a protective coating of non-digestible material around the organism, or the intracellular pathogens may release substances capable of blocking the lysozomal enzymatic machinery (Mauel, 1982). It was hoped that a study of biochemical aspects of macrophage function would clarify mechanisms of resistance of the Nocardia organism to host defence and contribute to the knowledge of mechanisms of pathogenesis of the nocardiae.

Macrophage activation is an essential part of the cellular immune response and evidence has already been presented that plasminogen activator, lysozyme release and morphological and ultrastructural changes truly reflect the state of macrophage activation. This study provides evidence of marked alterations in several macrophage functions during the first 21 days in the course of infection with N.asteroides and N.brasiliensis. The data show that macrophages are activated with respect to some parameters of macrophage activation in experimental murine N.asteroides and N.brasiliensis infections. In contrast, macrophage function was shown to be impaired by measurement of the index of macrophage function, lysozyme release. Inoculation of live organisms in the absence of adjuvants and assessment of macrophage activation in terms of microbicidal activity (Filice, Beaman and Remington, 1980) or in terms of cell cytoxicity (Krick and Remington, 1975) also gave rise to conflicting results. Morland and Kaplan (1977) state that activation is not a uniform and stereotyped response. Wing et al (1977) found that antimicrobial and antitumour effects of different populations of activated macrophages were

not necessarily mutually consistent (Hopper, Wood and Nelson, 1979). In this work it has been shown that macrophages from mice recently inoculated with pathogenic Nocardia are activated in terms of release of the enzyme plasminogen activator, morphology, ultrastructure and in vitro spreading activity, and that macrophage activation occurs early during experimental infection with N.asteroides and N.brasiliensis.

In addition, this investigation attempted to determine whether certain biochemical alterations in macrophage function occur in association with nocardial infections. This study demonstrates reduced intracellular levels and diminished secretion of lysozyme in vitro by Nocardia-activated macrophages at various time intervals post-inoculation. Davis-Scibienski and Beaman (1980a) suggested that maintenance of nocardial intracellular viability might be due to resistance to rather than avoidance of the hydrolytic environment and that organisms might be able to inactivate the contents of the lysozomes. Intracellular acid-phosphatase levels are shown to be reduced in N.asteroides infection (Black et al, 1983, 1985); and macrophage protein synthesis is reduced on ingestion of M.leprae, with consequent cessation of lysozyme synthesis (Ridley et al, 1985). From the results of the present study it appears that de novo synthesis of lysozyme is inhibited by Nocardia at certain times during infection. Black et al (1983) suggested that reduced acid-phosphatase activity suggests bacteria-induced macrophage dysfunction and that reduction of lysozomal enzyme levels correlates with the pathogenesis of intracellular parasites. Reduction in

intracellular lysozyme levels may facilitate intracellular survival of the Nocardia parasite and contribute to the pathogenesis of the disease.

This study examines the possibility that macrophage responses in N.asteroides and N.brasiliensis infections may be different by comparing biochemical, morphological and ultrastructural aspects of macrophage function in these infections. In terms of the aspects of macrophage function studied, major differences in macrophage responses to inoculation with N.asteroides and N.brasiliensis were not found, although minor differences in enzyme release were shown at certain times post-inoculation in the two types of infection. Production and release of lysozyme by peritoneal macrophages appears to be different at 13 and 21 days post-inoculation. Similarly, the degree of macrophage response in terms of plasminogen activator release appears to be different in N.asteroides and N.brasiliensis infections, although plasminogen activator release by macrophages is significantly enhanced over the 21 day period post-inoculation. Strain of micro-organism did not appear to influence morphology of macrophages, as judged by phase-contrast and SEM.

The implications of the findings in this study of macrophage function and its relationship to the histopathological features of the nocardial lesion are interesting, although reduction in macrophage intracellular levels of lysozyme has not entirely explained the differences in the pathogenesis and pathology of the two infections studied - lysozyme level is reduced in both

N.asteroides and N.brasiliensis infections at 7 days post-inoculation. This calls into question whether the original suggestion that the local pathogenic response to experimental murine infection with N.asteroides and N.brasiliensis might be quite different (Folb, Jaffe and Altmann, 1976). However, the evidence seems to be sound that the original observation was a valid one (Uesaka et al, 1971; Melendro et al, 1978; Ximenez et al, 1980; Rico et al, 1981; Conde et al, 1982). Another explanation might be that although a fairly broad series of probes has been used, that aspect of macrophage function that determines the difference in pathological response has not been identified. The third possibility is that the differences in pathological responses are explained by non-macrophage factors, eg lymphocytes, humoral factors, etc. This is difficult to accept because structurally and ultrastructurally it appeared that the difference between the two lesions resided in the macrophage. Therefore a study of the interelationships between reductions in macrophage intracellular lysozomal enzyme levels and the histopathological features of the lesion should clarify these findings.

What has been shown in the work reported here, and by others, is that the macrophage is definitely involved in murine responses to acute nocardial infections and that there appears to be a time sequence of biochemical events.

The histopathological studies by Folb, Jaffe and Altmann (1976) suggested that parameters of macrophage function may be depressed

in N.brasiliensis infection. This was shown to be so with respect to lysozyme release at 7 and 13 days post-inoculation. However, lysozyme release was also inhibited at 7 and 21 days post-inoculation with N.asteroides, indicating depression of macrophage function in infections due to this strain as well. The alternative idea that degree of activation or inhibition of macrophage function may be different in N.asteroides and N.brasiliensis infections appeared to be correct with regard to release of plasminogen activator and lysozyme.

We originally concerned ourselves with the study of Nocardia infections because we saw them as opportunistic in certain cases. It appears that all opportunistic infections are characterised by an extensive macrophage response in the body. In addition, there are striking parallels between the related organisms Nocardia and M.leprae, e.g. they are both facultative intracellular parasites, both are identifiable in macrophages, both produce different forms of pathological response and both reduce intracellular lysozyme levels. The consensus of opinion is that cell-mediated immunity is diminished in lepromatous leprosy (Rojas-Espinosa et al, 1982). It is likely that one mechanism of immunosuppression associated with opportunistic infections operates through suppression of macrophage function. This study, as in others, has shown that an aspect of macrophage function is impaired in experimental murine Nocardia infections. However, pathogenicity of Nocardia organisms and host defence consist of a complex and multiple series of factors (section 1.8). Reduction in intracellular lysozyme level and diminished secretion is one of

the factors which may contribute to the pathogenicity of the disease.

There are several limitations in the work presented in this dissertation:

(1) The study is necessarily selective because it has not been possible to encompass the full spectrum of macrophage responses.

(2) It has not been possible to use *in vitro* systems that examine more acutely the reactions between various components of cell-mediated immunity - for instance, systems to separate lymphocyte – macrophage interactions were not employed.

(3) The findings of this study in the mouse model can not necessarily be extrapolated to the human system. There is no proof that these two nocardial infections produce pathology in humans comparable to that produced in the mouse model.

However, the macrophage clearly has a significant place in host response to *Nocardia* infections.

If the hypothesis that modulation of biochemical function of macrophages by *Nocardia* infections is related to the differences in histopathology of the two lesions is correct, perhaps more

vigorous investigation of lymphocyte function and interactions with macrophages needs to be undertaken to elucidate the differences in the pathogenesis of the two infections studied.

6.2 SUMMARY OF FINDINGS OF THIS STUDY

1. Release of macrophage plasminogen activator:

1.1 Murine peritoneal macrophages exhibit significantly increased *in vitro* secretion of plasminogen activator in both *N.asteroides* and *N.brasiliensis* infections. Secretion of plasminogen activator was significantly elevated at 2, 7, 13 and 21 days post-inoculation compared with controls. It follows that macrophages from mice recently infected with the pathogens *N.asteroides* and *N.brasiliensis* are activated in terms of this parameter of macrophage activation.

1.2 Macrophage responses to both *N.asteroides* and *N.brasiliensis* appear to be modified over the 21 day period of infection with respect to plasminogen activator release. Macrophage function in *N.asteroides* and *N.brasiliensis* infections is similar in that release of plasminogen activator is elevated in both infections.

1.3 However, the pattern of secretion of plasminogen activator in *N.asteroides* infection appears to be different to that in *N.brasiliensis* infection. Therefore the alternative idea that the degree of macrophage activator may be different in *N.asteroides* and *N.brasiliensis* infections appears to be

correct with regard to the release of plasminogen activator.

1.4 Macrophage activation in terms of release of plasminogen activator occurs early in infection, that is at 2 days post-inoculation.

2. Intracellular lysozyme levels and release of the enzyme.

2.1 Secretion of lysozyme was significantly reduced in both N.asteroides and N.brasiliensis infections at 7 days post-inoculation, in N.brasiliensis infection at 13 days post-inoculation and in N.asteroides infection at 21 days post-inoculation. The functional status of macrophages is impaired at certain intervals post-inoculation.

2.2 This study demonstrates reduced intracellular levels as well as diminished secretion of lysozyme in vitro by Nocardia-activated macrophages at various time intervals post-inoculation.

2.3 It appears that de novo synthesis of lysozyme is inhibited by Nocardia at certain times during infection.

2.4 Production and release of lysozyme by peritoneal macrophages appears to be different in experimental N.asteroides and N.brasiliensis infections at 13 and 21 days post-inoculation and possibly at 2 days post-inoculation.

2.5 Macrophage responses to infections with N.asteroides and N.brasiliensis are modified over the 21 day infection period with respect to release of lysozyme.

2.6 Impairment of macrophage function in terms of reduced intracellular levels and diminished secretion of lysozyme may be one of the mechanisms of pathogenesis of the nocardiae.

3. Morphological and ultrastructural aspects of macrophage function.

3.1 Morphological and ultrastructural characteristics of activated macrophages were exhibited by macrophage from Nocardia-infected mice at each time interval post-inoculation studied. Features of activated macrophage were shown using both phase-contrast and scanning electron microscopy.

3.2 There did not appear to be any differences in the morphology or ultrastructure of N.asteroides- and N.brasiliensis -activated macrophages whether viewed using phase-contrast or SEM.

3.3 Duration of infection did not appear to influence cell morphology.

3.4 Enhanced *in vitro* spreading activity was observed in cultures of macrophages from *Nocardia*-infected mice throughout the period of study.

3.5 Macrophage activation in terms of morphological characteristics and spreading ability occurs early in infection, that is at 2 days post-inoculation.

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